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SUMITHRIN IMMUNOTOXICITY IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*) UPON EXPERIMENTAL EXPOSURE

MILTON LEVIN,[1]* BRUCE BROWNAWELL[2] AND SYLVAIN DE GUISE[1]

[1]*Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, U-89, Storrs, Connecticut 06269;* [2]*Marine Sciences Research Center, Stony Brook University, Stony Brook, New York 11794*

ABSTRACT An unusual mortality event in the fall of 1999 was observed in Long Island Sound lobsters resulting in the reduction of up to 99% of lobster landings. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried West Nile virus, an emerging disease in North America at that time. To determine the possible implications of pesticide application as a direct or contributing factor in the die-off, studies were conducted to determine the effects of experimental exposure to sumithrin on the health of lobsters. Lobsters were exposed in 20-gallon tanks for 5-d or 28-d, and the direct toxicity, as well as sublethal effects on the immune system was determined. The 96-h LC50 for sumithrin upon single exposure, as well as the 28-d LC50 for sumithrin upon repeated exposure, were greater than 1.0 µg/L, the highest concentration tested. No modulation of immune function was detected after sumithrin exposure for either the single or repeated exposures. Water concentrations of sumithrin decreased rapidly over the 5-d single sumithrin exposure, with a half-life of less than 2-h, with similar decay rates for all concentrations tested. Overall, this study showed that sumithrin degrades rapidly in salt water and did not exert significant toxicity in lobsters (it neither killed lobsters nor induced immunotoxicity) at the concentrations tested. It is unlikely that sumithrin significantly contributed to the 1999 lobster die-off in Long Island Sound.

INTRODUCTION

An unusual mortality event was observed in Long Island Sound (LIS) lobsters (*Homarus americanus* H. Milne Edwards, 1837) in the fall of 1999, resulting in a reduction of 90% to 99.9% of landings in western LIS and 60% to 80% in central and eastern LIS. The lobsters examined suffered from a *Paramoeba* sp. infection not previously described in lobsters, which mainly affected the nervous system (Mullen et al. 2004). Nevertheless, it is not known if this organism is solely responsible for the mortality event or represents a terminal opportunistic infection. The die-off corresponded in time with the application of pesticides, including sumithrin, for the control of mosquitoes that carried West Nile virus, a new emerging disease in North America at the time. Initial analyses for the presence of pesticides used for the control of West Nile virus-infected mosquitoes did not reveal detectable concentrations in randomly sampled lobsters, but these chemicals are relatively short lived and could have been metabolized. The assay results could not determine with certainty if LIS lobsters had been exposed to those pesticides. It is also possible that exposed lobsters could have suffered short- or long-term effects after the chemicals had been metabolized and were no longer detectable.

The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most susceptible and sensitive systems to the effects of xenobiotics. It is therefore highly possible that environmental stressors (chemical or others) that would have affected the immune system of lobsters in LIS would have rendered them more susceptible to infections (for example with paramoeba) and have played a significant role in the 1999 LIS lobster die-off.

Two pesticides used to control mosquitoes that carried West Nile virus, resmethrin and malathion, have recently been documented to reduce lobster hemocyte phagocytosis, an important innate immune response necessary to eliminate invading pathogens, upon experimental exposure (De Guise et al. 2005, De Guise et al. 2004). However, the immunotoxicity of sumithrin has not been documented in lobsters. This is of particular interest because the concentrations at which different pesticides are toxic to lobsters were recently compared with those that might have been present in Long Island Sound based on modeling and application data (Miller et al. 2005), but the modeling exercise had grouped sumithrin and resmethrin. It is therefore important to understand the toxicity of sumithrin in lobsters, relative to that of resmethrin.

To determine the possible implications of sumithrin application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to sumithrin on the health of lobsters.

MATERIALS AND METHODS

Experimental exposures were performed in aerated 20-gallon tanks each containing three lobsters, with a total of nine lobsters per concentration. Lobsters of both sexes were kept in the dark (except during sampling and water changes) in 15°C artificial seawater (Instant Ocean, Mentor, OH) at a salinity of 24‰ and exposed to technical grade sumithrin (Sigma, St. Louis, MO) using different regimes. The three concentrations of sumithrin tested were 0.01, 0.1, and 1.0 µg/L, in addition to unexposed controls.

Standard LC50 experiments were performed over the course of 96-h. Acute exposure lasted 5 d, with serial sampling on day 1, 3, and 5 and consisted of a single exposure to sumithrin (on day 0). Subacute exposure was performed over the course of 4 wk, with weekly serial sampling, followed by a weekly water change and re-exposure to an identical concentration of sumithrin as the original one. Experiments started with nine lobsters per concentration (except in a few instances when one lobster died during the acclimation period), which were all serially sampled until the end of the study. All experiments were conducted in the dark (except for sampling and water changes) to minimize possible breakdown of sumithrin

*Corresponding author. E-mail: milton.levin@uconn.edu

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through photodegradation. At the end of each exposure regimen, lobsters were euthanized using potassium chloride (Battison et al. 2000).

For the acute exposure, water samples (900 mL) were collected at time 0-h, 2-h, 4-h, 6-h, 12-h, 24-h, 72-h, and 120-h, using I-CHEM amber glass jars certified to meet or exceed "US EPA specifications and guidances for contaminant-free sample containers" (Nalgen Nunc International, Rochester, NY), prefilled with 25 mL hexane to initiate extraction and prevent microbial degradation of sumithrin. The samples were kept cold (4°C) and in the dark until analysis.

The sampling, extraction and HPLC-MS analysis of sumithrin followed the method outlined in Zulkosky et al. (2005). The method seems adequate for extracting dissolved sumithrin and sumithrin sorbed to suspended particles in water samples (Zulkosky et al. 2005). Samples were further shaken/extracted at SBU and hexane:water emulsions allowed to settle for up to a day, after which a fraction of the hexane was transferred with volumetric pipettes to a vial and gently evaporated to dryness under a stream of nitrogen gas. Samples were immediately redissolved in 1 mL of methanol, which was then analyzed directly by HPLC-MS. The analysis of sumithrin by HPLC-Tof-MS analyses was conducted with a Micromass LCT, equipped with a Waters 2695 HPLC and a Z-spray electrospray ionization source according to Zulkosky et al. (2005) Injection volumes were kept at 10 µL. The mobile phase was methanol: water with gradient elution varying from 40% to 95% methanol in 12 min and held at 95% for 4 min. The aqueous mobile phase contained 10 µM sodium acetate, and all sumithrin was analyzed from reconstructed ion chromatogram (signal to noise enhanced by using a small 40 mDa window around the actual accurate mass of sumithrin) as sodium adducts of parent molecules $(M + Na)^+$. Leucine enkephalin was added through postcolumn infusion to serve as an internal mass calibrant, to confirm analyte identification using accurate mass estimation (Benotti et al. 2003) And to allow for more accurate quantification using proprietary all file accurate mass (AFAM) correction software supplied by Waters/Micromass in their MassLynx software. The internal standard added prior to injection was *d*-6-malathion.

Quantitation was conducted by summing the area of two resolved sumithrin peaks consisting of cis- and transsumithrin, normalizing the area response to that of internal standard, and compared with 6 point calibration curves run daily with each

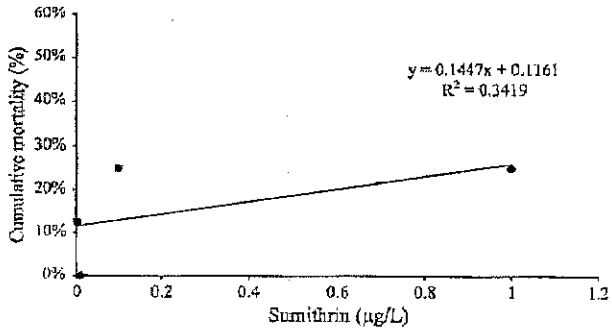

Figure 1. The LC_{50} of sumithrin was determined in lobsters after a single exposure followed by a 4-day observation and determination of mortality. The study started with eight lobsters in each concentration group.

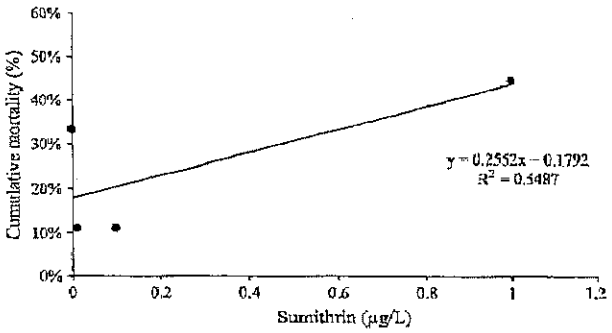

Figure 2. The LC_{50} of sumithrin was determined in lobsters after 4 weekly repeated exposures and determination of mortality. The study started with nine lobsters in each concentration group.

batch of samples. During an intercalibration exercise conducted at two concentrations with certified spike solutions, the SBU method resulted in data that agreed with the expected concentrations and with each of the other participating laboratories within approximately 20% in all cases. Analytical precision in that and other studies has been between 4% and 10% for samples extracted and analyzed separately on the same day.

The endpoint tested was the evaluation of the immune system using hemocyte phagocytosis on hemolymph samples. Briefly, 1.0 mL of hemolymph was collected from the dorsal vasculature and immediately transferred to a Vacutainer tube (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD). This proved to be the best anticoagulant for use with lobster hemolymph cells in preliminary studies in our laboratory. Phagocytosis was evaluated as previously described (De Guise et al. 2005, De Guise et al. 2004). Briefly, hemocytes were incubated in their hemolymph at room temperature (20°C to 25°C). Fluorescent latex beads (1 µm diameter) (Molecular probes, Eugene, OR) were diluted 1:10 in phosphate-buffered saline (PBS) and 25 µL of the bead mixture was added to 1 mL hemolymph. After a 1-h incubation in the dark, the fluorescence

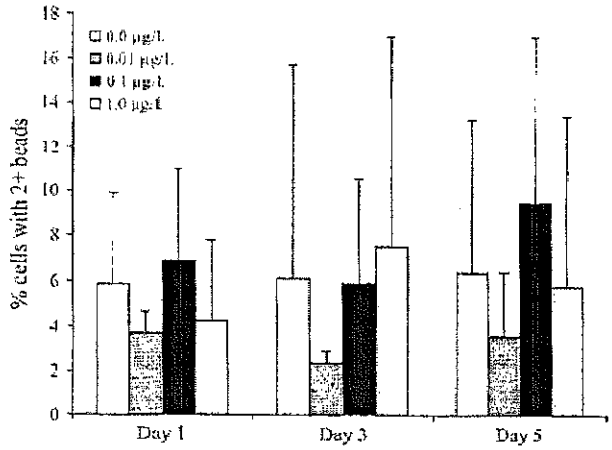

Figure 3. Phagocytosis of lobster cells as measured 1, 3, and 5 days after a single exposure to three different concentrations of sumithrin. Results are reported as mean + standard deviation (n = 7, 8, 8 and 7; 7, 8, 7, and 7; 6, 8, 5, and 6 for the 0, 0.01, 0.1, and 10.0 µg/L groups, respectively, on days 1, 3, and 5).

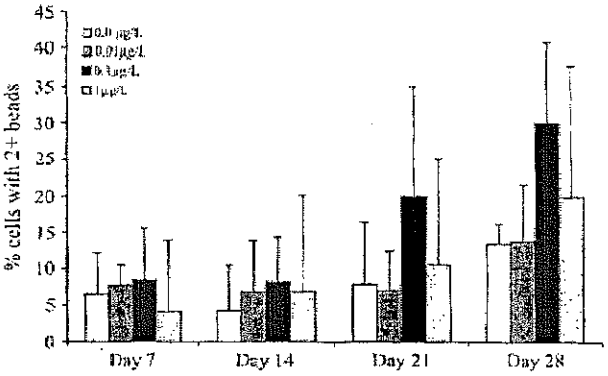

Figure 4. Phagocytosis of lobster cells as measured 7, 14, 21, and 28 days after weekly repeated exposure to three different concentrations of sumithrin. Results are reported as mean + standard deviation (n = 9, 8, 9, and 7; 8, 8, 8, and 7; 8, 8, 8, and 5; 6, 8, 8, and 5 for the 0, 0.01, 0.1, and 10.0 µg/L groups, respectively, on days 7, 14, 21, and 28).

of approximately 20,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer, using a forward scatter threshold to eliminate free beads from the acquisition. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized two or more beads. Results are reported as mean ± standard deviation. A one-way analysis of variance (ANOVA) with Dunnett test was used to compare the different experimental groups to the unexposed control group, using $p < 0.05$ for statistical significance. Regression analysis for the determination of the LC50 was performed using the Microsoft Excel software.

RESULTS

Direct Toxicity

The concentration of sumithrin that killed 50% of the exposed lobster (LC_{50}) was determined for both the 96-h and 28-d exposures. To do so, lobster mortality was recorded daily and cumulated over either 96-h or 28-d. The cumulative mortality was plotted against the concentrations of sumithrin

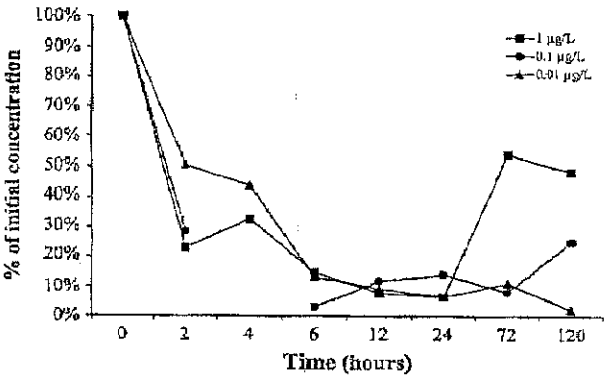

Figure 5. Degradation of sumithrin after 5 days (acute exposure), expressed as a percentage of the initially measured concentration. Note that a data point is missing for the 4-h time point at 0.1 µg/L, because the bottle broke during transport.

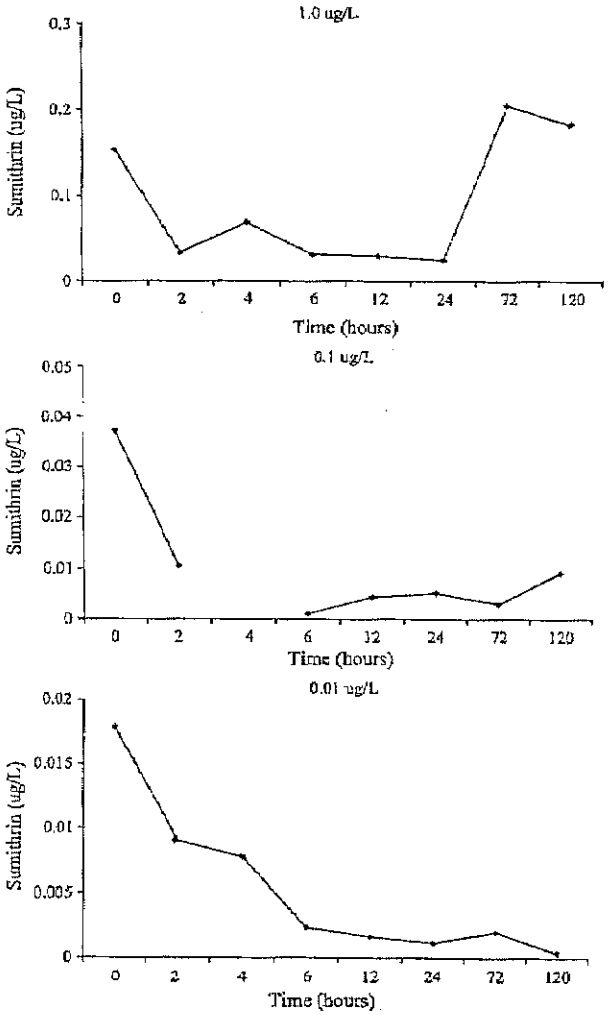

Figure 6. Changes in the three (1.0, 0.1, and 0.01 µg/L) target doses of sumithrin over 5 days (acute exposure). Note that a data point is missing for the 4-h time point at 0.1 µg/L, because the bottle broke during transport.

used (nominal concentration) and a linear regression curve was determined using the Microsoft Excel software. The LC_{50} (concentration of sumithrin when the curve crosses the 50% mortality) was calculated using the equation determined by the software for the regression curve (using $y = 50\%$). The 96-h and 28-d LC_{50}'s were both above 1.0 µg/L (nominal concentration), the highest concentration used in this study (Fig. 1, Fig. 2).

Immune Function

Phagocytosis was not significantly modulated in either the 5-d (Fig. 3) or 28-d (Fig. 4) exposure.

Analytical Toxicology

Water samples were analyzed to assess the degradation of sumithrin in the conditions used. The concentrations at time 0 (immediately after dosing) were 0.352, 0.038, and 0.018 µg/L for nominal concentrations of 1.0, 0.1 and 0.01 µg/L, respectively.

Concentrations generally decreased rapidly, reaching 50% of the initial concentration before or at the 2-h initial resampling (half life of 2-h or less, Fig. 5). The concentrations of sumithrin in time for the different nominal doses are shown in Figure 6. Note that a data point is missing for the 4-h time point at 0.1 µg/L, as the bottle broke during transport.

DISCUSSION

The current study assessed the toxicity of sumithrin in lobsters in view of the use of sumithrin around Long Island Sound to control West Nile virus-infected mosquitoes and its potential role in the 1999 lobster die-off.

In the current study, the 96-h LC_{50} for sumithrin was above 1.0 µg/L. The 96-h LC_{50} for resmithrin, another pyrethroid pesticide used to control mosquitoes and tested for its toxic potential in a previous study in lobsters, was also above 1.0 µg/L (De Guise et al. 2005). The 96-h LC_{50} for sumithrin in small crayfish, large crayfish, brown trout, and brook trout were 0.35, 0.71, 10.5, and 18.5 µg/L, respectively (Paul & Simonin 2006, Paul et al. 2005), with the lower two concentrations within the range of concentrations tested in the current study. Lobsters in this study were therefore less sensitive than crayfish to the deleterious effects of sumithrin. The 28-d LC_{50} for lobsters in this study was greater than 1 µg/L, the greater concentration tested. It seems that sumithrin was less toxic to lobsters than resmethrin, for which a 14-d LC_{50} in similar conditions was 0.75 µg/L (De Guise et al. 2005).

The evaluation of phagocytosis by flow cytometry is a sensitive indicator to measure the subtle sublethal effects of pesticides in lobsters (De Guise et al. 2005, De Guise et al. 2004). No modulation of immune function was detected after sumithrin exposure either for single or repeated exposures. It seems that sumithrin is less immunotoxic to lobsters than resmethrin, which at similar concentrations, significantly reduced phagocytosis on single and repeated exposures in similar experimental conditions (De Guise et al. 2005).

The concentrations of sumithrin measured in the water at time 0 (immediately after dosing) were less than half the nominal concentrations (what we know we put in the water) at the high and medium dose, and almost twice the nominal concentration at the low dose. All events could be explained by incomplete mixing of the sumithrin in the tanks, followed by sampling of a sample of water that may either overestimate or underestimate the concentration to be reached on complete mixing. In addition, it is possible that sumithrin rapidly adsorbed to either the walls of the tanks, the lobsters or organic matter present in the tank, yielding lower concentrations than expected in the water. The rise in concentrations towards the end of the experiment, particularly at the high dose, occurred with a simultaneous increase in organic particles in the water, to which sumithrin may have adsorbed. Despite the variations observed between nominal and measured concentrations, nominal concentrations were used throughout this manuscript for comparison with the resmethrin study (De Guise et al. 2005), which also used nominal concentrations. Overall, whatever the initial concentration of sumithrin, the decay rates were rather similar, with a half-life of less than 2-h in our sea water system.

A modeling exercise suggested the maximum concentrations of pyrethroids (the sum of resmethrin and sumithrin) reaching the bottom waters of Long Island Sound (maximum calculated 24-h average) was 0.082 µg/L (Miller et al. 2005), a concentration within the range of those tested in this study and that did not induce significant increases in mortality or deleterious effects on lobster immune functions.

Overall, our study showed that sumithrin degrades rapidly in salt water and did not exert significant toxicity in lobsters (it neither killed lobsters nor induced immunotoxicity). It was also less toxic than resmithrin, which was previously tested in our laboratory at the same doses, using the same assays. Overall, it is unlikely that sumithrin significantly contributed to the 1999 lobster die-off in Long Island Sound.

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Malathion immunotoxicity in the American lobster (*Homarus americanus*) upon experimental exposure

Sylvain De Guise [a,*], Jennifer Maratea [a], Christopher Perkins [b]

[a] *Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, U-89, Storrs, CT 06269, USA*

[b] *Environmental Research Institute, University of Connecticut, 270 Middle Turnpike, Unit 5210, Storrs, CT 06269, USA*

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Abstract

A lobster die-off reduced the 1999 fall landings in western Long Island Sound by up to more then 99%. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried West Nile virus, a new emerging disease in North America at the time. In order to determine the possible implication of pesticide application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to malathion on the health of lobsters. Lobsters were exposed in 20 gallon tanks, and the direct toxicity as well as sub-lethal effects on the immune system were determined. The 96 h LC_{50} for malathion upon single exposure was 38 μg/l. Malathion degraded rapidly in sea water, with 65–77% lost after 1 day and 83–96% lost after 3 days. Phagocytosis was significantly decreased 3 days after a single exposure to initial water concentrations as low as 5 ppb, when measured water concentrations were as low as 0.55 ppb. Similarly, effects on phagocytosis were observed at 1, 2 and 3 weeks after the initiation of weekly exposures. Cell counts did not differ significantly upon exposure to malathion. Malathion was not detected in muscle and hepatopancreas of exposed lobsters. Evaluation of phagocytosis is a sensitive indicator of subtle sub-lethal effects of malathion, and relatively small concentrations of malathion (6–7 times lower than the LC_{50}) can affect lobster defense mechanisms.

Keywords: Malathion; Lobster; *Homarus americanus*; immunotoxicity; LC50

1. Introduction

An unusual mortality event was observed in Long Island Sound (LIS) lobsters in the fall of 1999, resulting in a reduction of 90–99.9% of landings in western LIS and 60–80% in central and eastern LIS. The lobsters examined suffered from a *Paramoeba* sp. infection not previously described in lobsters, which mainly affected the nervous system (Mullen et al., 2003). Nevertheless, it is not known if this organism is solely responsible for the mortality event or represents a terminal opportunistic infection. The die-off corresponded in time with the application of pesticides, including malathion, for the control of mosquitoes that

* Corresponding author. Tel.: +1-860-486-0850; fax: +1-860-486-2794.
E-mail address: sdeguise@canr.uconn.edu (S. De Guise).

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Office of Pesticide Programs. The primary changes from the EPA method is the use of capillary column techniques in lieu of the packed column specified in the methods, and the use of GC/MS instead of a flame ionization detector. EPA method 616 is based upon older techniques and the ERI improvements to the method allow for the identification and quantitation at lower levels. The method detection limit (MDL) for the analysis of the malathion in water was 0.1 ppb (μg/l). The MDL for the analysis in lobster tissues was 15 ppb.

The endpoints tested include evaluation of the immune system using hemocyte counts and phagocytosis on hemolymph samples. Briefly, 2.0 ml of hemolymph was collected from the dorsal vasculature and immediately transferred to a Vacutainer tube (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD). This proved to be the best anticoagulant for use with lobster hemolymph cells in preliminary studies in our lab. Cells were then counted using a hemocytometer and Trypan blue to determine viability. Phagocytosis was evaluated as previously described (De Guise et al., 1995) with some variations. Hemocytes were incubated in their hemolymph at room temperature (20–25 °C). One μm diameter fluorescent latex beads (Molecular probes, Eugene, OR) were diluted 1:10 in phosphate-buffered saline (PBS) and 5 μl of the bead mixture was added for every 200 μl of hemolymph. After a 1 h incubation in the dark, 200 μl of each cell suspension was analyzed by flow cytometry. The fluorescence of approximately 10,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer, using a forward scatter threshold to eliminate free beads from the acquisition. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized two or more beads.

Results are reported as mean ± standard deviation. A one-way analysis of variance (ANOVA) with Dunnett's test was used to compare the different experimental groups to the unexposed control group, using $P < 0.05$ for statistical significance. When the power of the analysis was below 0.80, the threshold suggested by the analysis software (Sigma Stats, Jandel Corp, San Rafael, CA), a student T-test was used to compare each mixture to the control. Regression analysis for the determination of the LC_{50} was performed using the Microsoft Excel software.

3. Results

The direct toxicity of malathion in lobster was determined through a standard 96-h LC_{50}, the calculated concentration that killed 50% of the animals. To do so, lobster mortality was recorded daily and cumulated over a 4 day exposure. The cumulative mortality was plotted against the concentrations of malathion used and a linear regression curve was determined using the Microsoft Excel software. The LC_{50} was calculated using the equation determined by the software for the regression curve. The 96-h LC_{50} was 38 μg/l (or ppb) upon single exposure (Fig. 1).

The concentration of malathion was measured in the water on day 0 (initial concentration), as well as on days 1 and 3 after a single initial exposure to evaluate its degradation. Malathion degraded rapidly in our system, with 65–77% lost after one day and 83–96% after 3 days (Fig. 2). The half-life of malathion in our system, irrespective of the initial concentration, was approximately 12 h. No malathion was detected in lobster tissues at the end of the 5 day exposure.

Phagocytosis was significantly decreased 3 days (but not 1 or 5) after a single exposure to an initial water concentration as low as 5 ppb, the lowest concentration tested (Fig. 3). The water concentration of malathion on day 3 was 0.55 ppb.

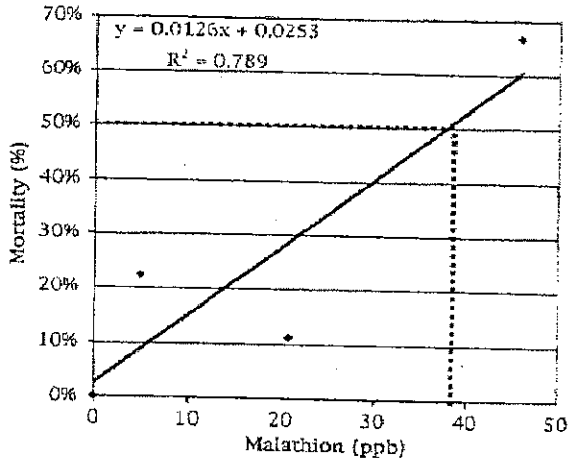

Fig. 1. The LC_{50} (dotted line) of malathion was determined in lobsters after a single exposure followed by a 4 day observation and determination of mortality. The study started with nine lobsters in each concentration group.

carried West Nile virus, a new emerging disease in North America at the time. Initial analyses for the presence of pesticides used for the control of West Nile virus-infected mosquitoes did not reveal detectable concentrations in randomly sampled lobsters, but these chemicals are relatively short lived and could have been metabolized. The assay results could not determine with certainty if LIS lobsters had been exposed to those pesticides. It is also possible that exposed lobsters could have suffered short- or long-term effects after the chemicals had been metabolized and were no longer detectable.

The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most susceptible and sensitive systems to the effects of xenobiotics. It is therefore highly possible that environmental stressors (chemical or others) that would have affected the immune system of lobsters in LIS would have rendered them more susceptible to infections (for example with paramoeba) and have played a significant role in the 1999 LIS lobster die-off. Malathion immunotoxicity has been documented in several species of laboratory animals including its effects on both humoral (diminution of the antibody response upon injection with tetanus toxoid, ovalbumin and sheep red blood cells) and cellular immune responses (marked inhibition of leucocyte and macrophage migration) of mice, rats and rabbits (Banerjee et al., 1998). The effects on macrophages, were also documented, including an increased respiratory burst by peritoneal macrophages upon stimulation after in vivo exposure to malathion (Rodgers and Ellefson, 1990), using a mechanism dependent on mast cells (Rodgers et al., 1996). Malathion was also documented to affect the natural and acquired immunity of fish (Japanese medaka), including a dose-dependent reduction of the production of antibodies to sheep red blood cells and a mild decrease in the superoxide production by kidney phagocytes, which resulted in a decrease in resistance to infection by *Yersinia ruckeri*, a common bacterial fish pathogen (Beaman et al., 1999). The lethal and immunotoxic effects of malathion on lobsters have never been determined. In order to determine the possible implication of malathion application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to malathion on the health of lobsters.

2. Material and methods

Experimental exposures were performed in aerated 20 gallon tanks each containing three lobsters, with a total of nine lobsters per concentration. The three concentrations of malathion tested were 5, 21 and 46 ppb, in addition to unexposed controls. Lobsters from Maine were kept in the dark (except during sampling and water changes) in 14 °C artificial sea water (Instant Ocean, Mentor, OH) at a salinity of 24‰ and exposed to technical grade malathion (Fisher Scientific, Pittsburgh, PA) using different regimes. Standard LC_{50} experiments were performed over the course of 96 h. Acute exposure lasted 5 days, with serial sampling on day 1, 3 and 5, and consisted of a single exposure to malathion. Subacute exposure was performed over the course of 4 weeks, with weekly serial sampling, followed by a weekly water change and re-exposure to an identical concentration of malathion as the original one. All experiments started with nine lobsters per concentration, which were all serially sampled until the end of the study.

At the end of each exposure regime, lobsters were sacrificed and a gross and histopathological examination was performed to determine the presence/absence of pathological lesions, and to determine whether or not they were associated with exposure to malathion. Lobsters were euthanized using potassium chloride (Battison et al., 2000), and a complete necropsy was performed. Tissues were fixed in 10% neutral buffered formalin. Select tissues were then transferred to Dietrichs with 5% trichloroacetic acid for 48 h for partial decalcification of exoskeleton. Tissues were then trimmed and processed for paraffin embedding. Tissues were sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined by light microscopy for the presence/absence of lesions.

Muscle and hepatopancreas were also collected and frozen during the post-mortem exam to analyze for the presence of malathion. Water samples (1 l) were collected periodically using I-CHEM amber glass jars certified to meet or exceed "US EPA specifications and guidances for contaminant-free sample containers" (Nalgen Nunc International, Rochester, NY). Water and tissue samples were analyzed at the Environmental Research Institute (ERI) based upon a modified form of EPA Method 616. This EPA method is not validated for sediment and tissue from the EPA

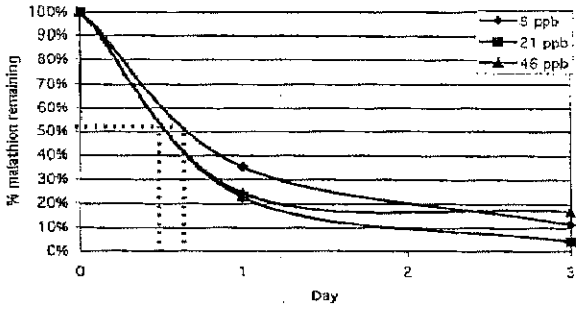

Fig. 2. Degradation of malathion in artificial sea water at 14 °C over a five day period, using three different initial concentrations. The half-life of malathion (dotted lines) was approximately half a day, irrespective of the initial concentration.

Phagocytosis was also significantly affected in the course of the sub-acute (month-long) exposure (Fig. 4). There was a significant reduction of phagocytosis one week after the initial exposure to 21 ppb, and 2 weeks after the initial exposure to 5 ppb. There was a significant reduction of phagocytosis at all concentrations tested 3 weeks after the initial exposure. On week 4, while seven animals remained in the control group, only one, three and two animals remained for the 5, 21 and 46 ppb exposure group, respectively, due to mortalities. Statistical comparison with the control group was therefore not possible for two of the three exposure groups, and the 21 ppb exposure group was not significantly different from the unexposed control group.

Cell counts did not differ significantly upon exposure to malathion. Gross or histological lesions were not observed upon exposure to malathion.

4. Discussion

While malathion is only slightly toxic to mammalian species at relatively high concentrations, its toxicity is generally much higher in aquatic species. Malathion has a wide range of toxicities in fish, extending from very highly toxic in the walleye (96-h LC_{50} of 0.06 mg/l) to highly toxic in brown trout (0.1 mg/l), rainbow trout (0.25 mg/l) and cutthroat trout (0.28 mg/l), moderately toxic in Nile tilapia (4.6 mg/l) and fathead minnow (8.6 mg/l) and slightly toxic in goldfish (10.7–11.3 mg/l), mosquitofish (0.70–12.68 mg/l) and topmouth gudgeon (14.5 mg/l) (Johnson and Finley, 1980; Kidd and James, 1991; Tietze et al., 1991; U.S. Public Health Service, 1995; Shao-nan and De-fang, 1996). The toxicity of malathion for aquatic or marine crustaceans in a 96-h bioassay ranged from 1 to 280 μg/l (Forget et al., 1998). Lobsters, with a LC_{50} of 38 ppb or 0.038 mg/l, appear to be very sensitive to the acute lethal effects of malathion compared to other aquatic species, with a LC_{50} half of that for the walleye, the most sensitive fish species, and within the relatively wide range of toxicity for aquatic and marine crustaceans. It is interesting to note that our study, as others, evaluated the toxicity of technical grade malathion, and that

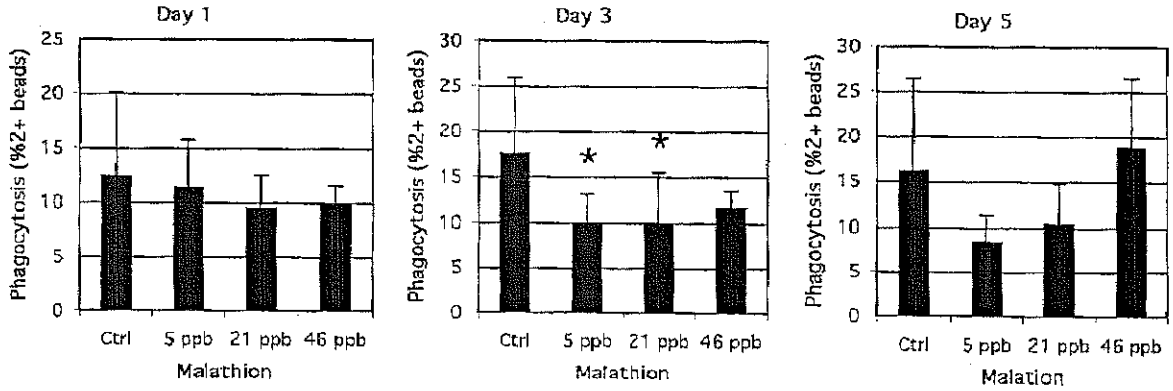

Fig. 3. Phagocytosis of lobster cells as measured 1, 3 and 5 days after a single exposure to three different concentrations of malathion. Results are reported as mean ± standard deviation (n = 9, 8, 9 and 7; 9, 7, 8 and 3; 9, 7, 6 and 2 for the control, 5, 21 and 46 ppb groups, respectively, on days 1, 3 and 5). Symbol asterisk (*) significantly different from unexposed control ($P < 0.05$).

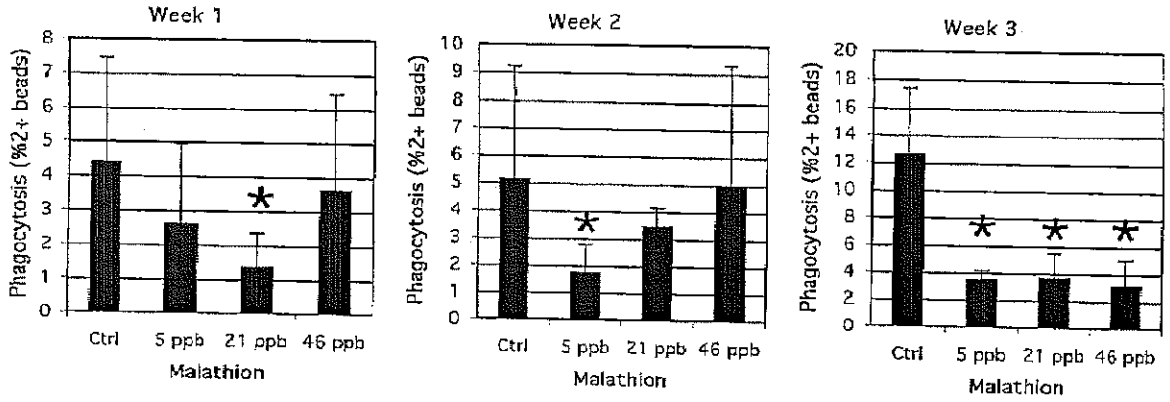

Fig. 4. Phagocytosis of lobster cells as measured on days 7, 14, 21 and 28, in each case 7 days after a weekly exposure to three different concentrations of malathion. Results for week 4 are not shown since only one, three and two animals remained for the 5, 21 and 46 ppb exposure group, respectively. Results are reported as mean ± standard deviation ($n = 9, 4, 4$ and 9; 9, 3, 3 and 6; 9, 2, 3 and 3 for the control, 5, 21 and 46 ppb groups, respectively, on weeks 1, 2 and 3). Symbol asterisk (*) significantly different from unexposed control ($P < 0.05$).

one study demonstrated that commercial formulations of malathion were 1.176 times more toxic than the technical material (Haider and Inbaraj, 1986).

The breakdown of malathion in our system was very rapid, with a half-life of approximately 12 h. This is in contrast with raw river water, for which the half-life is less than 1 week, and distilled water, in which malathion remained stable for 3 weeks (Howard, 1991). In general, it was determined that persistence of the pesticides decreases with increase in temperature, pH and organic content (Kaur et al., 1997). In sterile seawater, the degradation increases with increased salinity (Howard, 1991). We performed our experiments in a static/static renewal mode rather than flow through system to better mimic the sporadic environmental exposure to pesticides. Also, we chose to conduct our experiments at a temperature (14 °C) and salinity (1.024) at which lobsters were comfortable, to minimize stress and to ensure that we did not overestimate the toxicity of malathion in lobsters. Also, experiments were conducted in the dark (except for sampling and water changes) to avoid excessive breakdown of malathion through photodegradation.

Because of its very short half-life, malathion is not expected to bioconcentrate in aquatic organisms. However, brown shrimp showed an average concentration of 869 and 959 times the ambient water concentration in two separate samples (Howard, 1991). This was not the case in lobsters from this study, for which malathion was not detected in tissues 5 days after exposure. This could be the result of complete metabolism in lobster tissues or the inability to detect malathion due to a lack of sensitivity of the analytical methods. Nevertheless, the very rapid breakdown of malathion in our system suggests that failure to measure malathion in water samples or tissues does not necessarily mean lack of exposure. At day 3 of our acute exposure study, the concentrations of malathion in the water were very low, yet effects on phagocytosis were demonstrated in exposed lobsters in which no malathion was detected in tissues.

Effects on lobster hemocyte phagocytosis were observed at relatively low concentrations, sometimes without effects at higher concentrations. Immunotoxicological effects not following a dose–response relationship have been documented before in laboratory animals. For example, Lawrence (1981) documented an increase in humoral response to sheep erythrocytes and T lymphocyte activity in mice exposed to 0.08 and 0.4 mM lead acetate for 4 weeks, while exposure to 2.0 mM did not induce significant changes, and exposure to 10.0 nM resulted in a suppression of these functions. Similarly, mitogen-induced lymphocyte proliferation in mice exposed to lead acetate for four weeks was significantly enhanced upon exposure to 0.4 and 0.08 mM for ConA and PHA, respectively, while exposure to 2.0 and 10.0 mM had no significant effect (Lawrence, 1981). In another study, exposure to

10 and 250 ppm cadmium chloride for 90 days significantly reduced mouse lymphocyte proliferation upon stimulation with PHA, Con A or LPS, while exposure to 50 ppm did not result in significant modulation of the response (Thomas et al., 1985).

Our data suggest that evaluation of phagocytosis using flow cytometry is a sensitive indicator of subtle sub-lethal effects of malathion, and that transient exposure to relatively small concentrations of malathion (6–7 times lower than the LC_{50}) can affect lobsters defense mechanisms, even with rapidly decreasing water concentrations. These results are not surprising given that the immunotoxicity of malathion on macrophages as well as natural and acquired immunity has been documented in several species of laboratory animals (Rodgers and Ellefson, 1990; Rodgers et al., 1996; Banerjee et al., 1998) and in fish (Beaman et al., 1999). Nevertheless, it is interesting to note that the initial water concentrations that resulted in immunotoxicity in lobsters (5 ppb or 5 μg/l) are 40 times lower that those which resulted in reduced immune functions and 20 times lower than those which resulted in reduced resistance to a pathogen in a fish study (Beaman et al., 1999). Lobsters appear extremely sensitive to the immunotoxic effects of malathion. Various aquatic invertebrates have been documented as extremely sensitive to sublethal effects of malathion, with EC_{50} values from 1 μg/l to 1 mg/l (Menzie, 1980). Our immunotoxicity results place lobsters at the most sensitive end of the spectrum, with significant effects on the population (not an EC_{50}) at 5 μg/l. Mammalian studies comparing the relationship between immune functions and host resistance showed a good correlation between changes in the immune tests and altered host resistance, with no instances where host resistance was altered without affecting an immune test (Luster et al., 1993). We have documented immunotoxic effects in lobsters on phagocytosis, reported as the most important defense mechanism in all phyla of the animal kingdom (van Oss, 1986). Given that defective phagocyte function is an important cause of increased susceptibility to opportunistic pathogens in mammalian species (Rotrosen and Gallin, 1987), and the above referenced data in mammalian immunotoxicology (Luster et al., 1993), it is likely that malathion-induced suppression of phagocytosis in lobsters could result in a significant decrease in lobster resistance to disease.

The current study assessed the toxicity of malathion in lobsters in view of the use of malathion around Long Island Sound to control West Nile virus-infected mosquitoes and its potential role in the 1999 lobster die-off. We documented that lobsters appear to be highly sensitive to the toxic effects of malathion compared to other species for which literature was available. However, to the authors' knowledge, no data have documented the actual exposure of lobsters to malathion upon environmental application, and it is not known if the concentrations that we documented to be toxic to lobsters were ever found in their environment in the course of the die-off. It is therefore not possible to determine with any certitude the role of malathion in the 1999 lobster die-off. Our results on the toxicity of malathion to lobster can nevertheless be useful in risk assessment, and our upcoming results on the toxicity of methoprene and resmethrin will help in making informed decisions on further use of pesticides for application around Long Island Sound.

5. Conclusion

In conclusion, our results suggest that lobsters are highly sensitive to both the lethal and sub-lethal toxicity of malathion in sea water. A reduction in immune functions could likely result in an increase susceptibility to infectious agents, and could have contributed to the mass mortality if exposure was sufficient.

Acknowledgements

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University of Connecticut
Connecticut Sea Grant College Program

Avery Point Campus

November 4, 2011

Roger Frate
Darien Seafood
1941 Post Rd
Darien, CT 06820

Dear Roger,

This letter is to follow up on our recent conversation on the toxicity of pesticides in lobsters. As we discussed, my lab was involved in the experimental assessment of the toxicity (lethal and sub-lethal) of different pesticides on adult lobsters under laboratory conditions (artificial seawater, salinity of 24‰, held at 14°C). A summary of those results follows.

The concentration of malathion that killed 50% of the lobsters (LC50) in our lab experiments was 38 µg/l, or parts per billion (ppb). The concentration that reduced their immune functions was 5 ppb, the lowest concentration tested in our lab. Water concentrations of malathion degraded rapidly in our system, with 65–77% lost after one day and 83–96% after 3 days. These results have been published in a peer-reviewed article (De Guise et al. 2004).

The concentration of resmethrin that killed 50% of the lobsters (LC50) in our lab experiments was 0.75 µg/l, or parts per billion (ppb). The concentration that reduced their immune functions was 0.01 ppb, the lowest concentration tested in our lab. These results have been published in a peer-reviewed article (De Guise et al. 2005).

The concentration of sumithrin that killed 50% of the lobsters (LC50) in our lab experiments was greater than 1 µg/l, or parts per billion (ppb), the highest concentration tested. Sumithrin did not affect immune functions in lobsters at concentrations as high as 1 ppb, the highest concentration tested in our lab. Water concentrations of sumithrin degraded rapidly in our system, with a half-life of less than 2 hours. These results have been published in a peer-reviewed article (Levin et al. 2007)

The concentration of methoprene that killed 50% of the lobsters (LC50) in our lab experiments was greater than 221 µg/l, or parts per billion (ppb), the highest concentration tested. The concentration that reduced their immune functions was 33 ppb. Water concentrations of methoprene degraded rapidly in our system, with a half-life of less than a day. These results have not yet been published in a peer-reviewed article, but the results have been presented in conferences and workshops. It is worth noting that methoprene bioaccumulated in lobster tissues such as hepatopancreas in our studies, a finding that was also reported by others, with the eye stalk accumulating concentrations

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of methoprene over 500 times higher than in the seawater they were in (Walker et al. 2005). The same authors also found that methoprene affects protein synthesis in lobsters (Walker et al. 2005).

Please understand that the studies cited above were performed to determine the concentrations of pesticides that adversely affected lobsters in laboratory conditions. These studies did not test whether or not lobsters in Long Island Sound encountered such concentrations of pesticides. My lab did not test water or lobster tissues in Long Island Sound for the presence of pesticides. This would require the expertise of others.

I hope that the information above provides clarifications on the effects of different pesticides in lobsters. I have enclosed copies of the relevant papers for your reference.

Yours,

Sylvain De Guise, DMV, PhD
Director, Connecticut Sea Grant

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Journal of Shellfish Research, Vol. 24, No. 3, 781–786, 2005.

RESMETHRIN IMMUNOTOXICITY AND ENDOCRINE DISRUPTING EFFECTS IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*) UPON EXPERIMENTAL EXPOSURE

SYLVAIN DE GUISE,[1]* JENNIFER MARATEA,[1] ERNEST S. CHANG[2] AND CHRISTOPHER PERKINS[3]

[1]*Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, U-89, Storrs, Connecticut 06269;* [2]*Bodega Marine Laboratory, University of California-Davis, PO Box 247, Bodega Bay, California 94923;* [3]*Environmental Research Institute, University of Connecticut, 270 Middle Turnpike, Unit 5210, Storrs, Connecticut 06269*

ABSTRACT A lobster die-off significantly reduced the 1999 fall landings in western Long Island Sound. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried West Nile virus, a new emerging disease in North America at the time. To determine the possible implication of pesticide application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to resmethrin on the health of lobsters. Lobsters (*Homarus americanus*) were exposed in 80-L tanks, and the direct toxicity as well as sublethal effects on the immune and endocrine system were determined. The 96-h LC_{50} for resmethrin on single exposure was greater than 1 μg/L, the highest concentration tested in our experiments, whereas the 14-day LC_{50} was 0.75 μg/L. Phagocytosis was significantly decreased 5 days after a single exposure to initial water concentrations of 1 and 0.1 μg/L, as well as after weekly exposure to 0.1 μg/L (week 3 and 4) and 0.01 μg/L (week 4). Cell counts varied widely and inconsistently upon exposure to resmethrin. Evaluation of phagocytosis is a sensitive indicator of subtle sublethal effects of resmethrin. Crustacean hyperglycemic hormone (CHH), a potential stress-related hormone in lobsters, was measured in the hemolymph of the chronically-exposed animals. Significant increases in CHH concentrations were observed after 4 wk of exposure to 0.1 μg/L. Whereas it is yet unknown if the concentrations at which toxicity was documented were ever encountered by lobsters in Long Island Sound during the 1999 die off, exposure resulting in the modulation of their immunology and physiology could likely have contributed to increasing lobster susceptibility to infectious diseases.

KEY WORDS: resmethrin, lobster, *Homarus americanus*, immunotoxicity, endocrine disruption, crustacean hyperglycemic hormone, CHH

INTRODUCTION

An unusual mortality event was observed in Long Island Sound (LIS) lobsters (*Homarus americanus* H. Milne Edwards, 1837) in the fall of 1999, resulting in a reduction of 90% to 100% of landings in western LIS. The lobsters examined suffered from a *Paramoeba* sp. infection not previously described in lobsters, which mainly affected the nervous system (Mullen et al. 2004). Nevertheless, it is not known if this organism was solely responsible for the mortality event or represented a terminal opportunistic infection. The die-off corresponded in time with the application of pesticides, including resmethrin, for the control of mosquitoes that carried West Nile virus, a new emerging disease in North America at the time. Initial analyses for the presence of pesticides used for the control of West Nile virus-infected mosquitoes did not reveal detectable concentrations in randomly sampled lobsters, but these chemicals are relatively short lived and could have been metabolized. The assay results could not determine with certainty if LIS lobsters had been exposed to those pesticides. It is also possible that exposed lobsters could have suffered short- or long-term effects after the chemicals had been metabolized and were no longer detectable.

The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most susceptible and sensitive systems to the effects of xenobiotics (Tryphonas & Feeley 2001). It is, therefore, highly possible that environmental stressors (chemical or others) that would have affected the immune system of lobsters in LIS would have rendered them more susceptible to infections (for example with paramoeba) and have played a significant role in the 1999 LIS lobster die-off. Resmethrin, especially when synergized by piperonyl butoxide as is the case in commercial preparations (Scourge), is a highly effective mosquito adulticide. Nevertheless, concern over its toxicity had the United States Environmental Protection Agency impose a restricted use classification on Scourge. Whereas the toxic effects (lethal and sublethal, upon acute and chronic exposure) of resmethrin have been evaluated in a range of aquatic species (Rand 2002, Holck & Meek 1987, Johnson & Finley 1980, EPA 1988, Tietze et al. 1991, Paul & Simonin 1996), including vertebrates and invertebrates, the lethal and sublethal (immunotoxic and endocrine disruptor) effects of resmethrin on lobsters have never been determined. To determine the possible implication of resmethrin application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to resmethrin on aspects of the health and physiology of lobsters.

MATERIALS AND METHODS

Experimental exposures were performed in aerated 80-L tanks each containing three lobsters, with a total of nine lobsters per dose. Lobsters of both sexes from Maine were distributed randomly between groups and kept in the dark (except during sampling and water changes) in 14°C artificial seawater (Instant Ocean, Mentor, Ohio) at a salinity of 24 parts per thousand and exposed to technical grade resmethrin (ICN Biomedicals Inc., Aurora, Ohio) using different regimes. Standard LC_{50} experiments were performed over the course of 96 h. LC_{50} was also calculated after the 14-day exposure, for which exposure occurred on day 0 and 7. Acute exposure lasted 5 days, with serial sampling on day 1, 3 and 5 and consisted of a single dose of resmethrin on day 0. Subacute exposure was performed over the course of 4 wk, with weekly serial sampling, followed by a weekly water change and re-exposure to an identical concentration of resmethrin as the

*Corresponding author. E-mail: sylvain.deguise@uconn.edu

2005
FIA states that it is possible but the chemicals are a factor in the cause of die off

original dose. All experiments started with nine lobsters per dose, which were all serially sampled until the end of the study. All experiments were conducted in the dark (except for sampling and water changes) to minimize possible breakdown of resmethrin through photodegradation. The concentrations of resmethrin for each exposure group were 0, 0.01, 0.1 and 1.0 μg/L and are referred to as the nominal (calculated) concentrations added to the water.

At the end of each exposure regimen, lobsters were sacrificed and a gross and histopathologic examination was performed to determine the presence/absence of pathologic lesions, and to determine whether they were associated with exposure to resmethrin. Lobsters were euthanized using potassium chloride (Battison et al. 2000), and a complete necropsy was performed. Tissues were fixed in 10% neutral buffered formalin. Select tissues were then transferred to Dietrichs with 5% trichloroacetic acid for 48 h for partial decalcification of exoskeleton. Tissues were then trimmed and processed for paraffin embedding. Tissues were sectioned at 4 μm, routinely stained with hematoxylin and eosin and examined by light microscopy for the presence/absence of lesions.

Muscle and hepatopancreas were also collected and frozen during the postmortem exam to analyze for the presence of resmethrin. Water samples (1 L) were collected periodically using precleaned I-CHEM amber glass jars certified to meet or exceed "US EPA specifications and guidance for contaminant-free sample containers" (Nalgen Nunc International, Rochester, New York). Water samples were preserved with methylene chloride at the time of collection. Water and tissue samples were analyzed at the Environmental Research Institute (ERI) based on a modified form of EPA method 616. This EPA method is not validated for sediment and tissue analysis by the EPA Office of Pesticide Programs. The primary changes from the EPA method is the use of capillary column techniques in lieu of the packed column specified in the methods, and the use of GC/MS instead of a flame ionization detector. EPA method 616 is based on older techniques and the ERI improvements to the method allow for the identification and quantitation at lower levels. The practical quantitation limit for the analysis of the resmethrin in water was 0.5 μg/L. The practical quantitation limit for the analysis in lobster tissues ranged from 0.2–1.8 mg/L.

The immunologic endpoints tested include evaluation of hemocyte counts and phagocytosis on hemolymph samples. Briefly, 2.0 mL of hemolymph was collected from the dorsal vasculature and immediately transferred to a Vacutainer tube (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD). This proved to be the best anticoagulant for use with lobster hemolymph cells in preliminary studies in our laboratory. Cells were then counted using a hemocytometer and Trypan Blue to determine viability. Phagocytosis was evaluated as previously described (De Guise et al. 2004a). Briefly, hemolymph (with anticoagulant as described earlier) was incubated at room temperature (20°C to 25°C). Fluorescent latex beads (1-μm diameter) (Molecular probes, Eugene, Oregon) were diluted 1:10 in phosphate-buffered saline (PBS) and 5 μl of the bead mixture was added for every 200 μl of hemolymph. After a 1 h incubation in the dark, 200 μl of each cell suspension was analyzed by flow cytometry. The fluorescence of approximately 10,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, California) flow cytometer, using a forward scatter threshold to eliminate free beads from the acquisition. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized 2 or more beads.

Hemolymph was obtained via syringe before addition of resmethrin and after chronic exposure. Crustacean hyperglycemic hormone (CHH) was quantified by enzyme-linked immunosorbent assay as previously described (Chang et al. 1999). Standards were from the crayfish *Orconectes limosus* and corrected for lobster equivalents. The polyclonal antisera were rabbit antiCHH.

Results are reported as mean +/− standard deviation. A separate 1-way analysis of variance (ANOVA) with Dunnett test was used to compare the different experimental groups to the unexposed control group at each time point, using $P < 0.05$ for statistical significance.

RESULTS

The direct toxicity of resmethrin in lobster was determined through a standard 96-h LC_{50}, the calculated concentration that killed 50% of the animals. Mortalities were not recorded in lobsters exposed to the highest concentrations of resmethrin used in this study (1 μg/L). It was then concluded that the 96-h LC_{50} of resmethrin in lobsters was greater than 1 μg/L. The 14-day LC_{50} calculated based on the cumulative mortality was 0.75 μg/L (Fig. 1).

The concentration of resmethrin was measured in the water on day 0 (initial concentration), as well as on day 1, 3 and 5 after a single initial exposure, to evaluate its degradation. On day 0, the resmethrin concentration measured in water was 1.08 μg/L. Resmethrin degradation in our system could not be measured accurately because concentrations were not detectable in most instances; most concentrations used were under the practical quantitation limit of 0.5 μg/L (data not shown). Nominal concentrations were then used throughout this study.

Phagocytosis was significantly decreased 5 days (but not 1 or 3) after a single exposure to an initial water concentration of 1 and 0.1 μg/L resmethrin, the two highest concentrations tested (Fig. 2). No resmethrin was detected in lobster tissues at the end of the 5-day exposure.

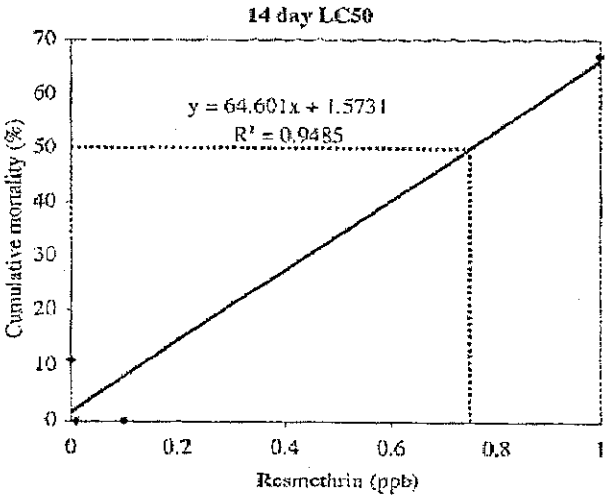

Figure 1. The 14-day LC_{50} (dotted line) of resmethrin was determined in lobsters after weekly exposures (day 0 and 7) followed by observation and determination of mortality. The study started with 9 lobsters in each concentration group.

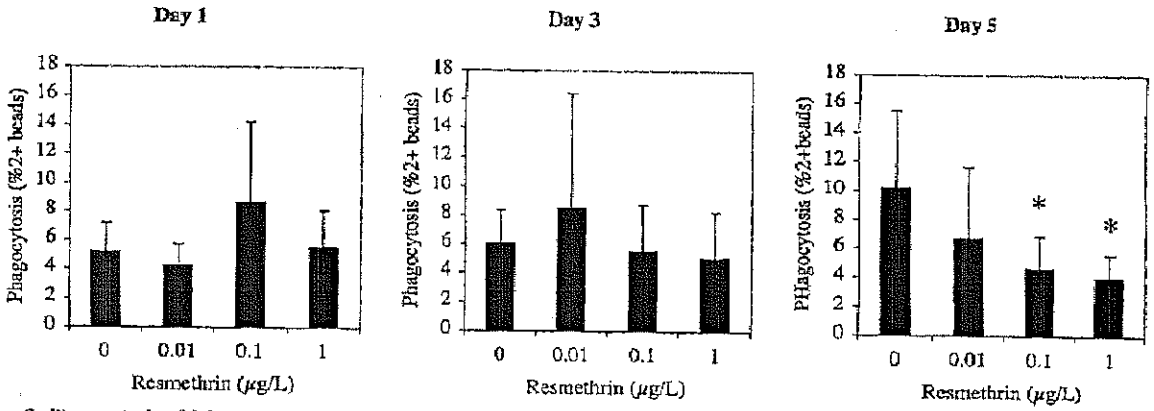

Figure 2. Phagocytosis of lobster cells as measured 1, 3 and 5 days after a single exposure to three different concentrations of resmethrin. *significantly different from unexposed control ($P < 0.05$).

Phagocytosis was affected significantly in the course of the subacute (month-long) exposure (Fig. 3). The highest concentration used in this study (1 μg/L) was omitted from week 2, 3 and 4 because mortality in that exposure group on those time points was 67%, 78% and 89%, respectively. There was a significant reduction of phagocytosis after exposure to 0.1 μg/L resmethrin (3 and 4 wk after initial exposure), and 0.01 μg/L (4 wk after initial exposure). No resmethrin was detected in lobster tissues at the end of the 4-wk exposure.

Cell counts varied widely and inconsistently in controls and upon exposure to resmethrin, with no significant differences from controls except for a significant reduction at the highest concentration on day 7 of the monthly exposure. Gross or histologic lesions were not observed when exposed to resmethrin.

Hemolymph concentrations of CHH were elevated significantly after 4 wk of exposure at 0.1 μg/L (Fig. 4). There seems to be a dose-response relationship, however the data for 0.01 μg/L are not significantly different from the controls.

DISCUSSION

The acute and chronic toxicity of resmethrin on aquatic organisms has been reviewed recently, with the conclusion that it was acutely toxic to fish and invertebrates in the 0.22–15.0 μg/L range (Rand 2002). This is in agreement with the findings of several previous studies in invertebrates (Holck & Meek 1987) and a wide range of fish species (Johnson & Finley 1980, EPA 1988, Tietze et al. 1991), with the exception of channel catfish in which acute toxicity occurred at a slightly higher concentration (16.6 μg/L) (Johnson & Finley 1980). The lack of acute lethal toxicity in a 96-h assay suggests that lobsters are not much more sensitive to resmethrin than other aquatic species. On the other hand, the toxicity of resmethrin became more evident over time, with a 14-day LC50 of 0.75 μg/L. It is important to note that our study tested the toxicity of technical grade resmethrin, and not the commercial preparations that are synergized with piperonyl butoxide, which was approximately four times more toxic (LC50 0.23 μg/L) than the technical grade compound (LC50 1.3 μg/L) in pink shrimp (Rand 2002). It is also possible that the present study underestimated the acute toxicity of resmethrin in lobsters because a static exposure was chosen, to better mimic pesticides application. Flow-through exposure resulted in a LC50 (0.22 μg/L) more than 10 times lower than static exposure (LC50 3.7 μg/L) in *Daphnia magna* (Rand 2002). Whereas other studies reported that acute toxicity occurred within 24 h (Rand 2002), significant deaths in this study only occurred at the highest concentration tested and after more than 1 wk. It is unlikely that the toxicity of resmethrin in lobsters was overestimated in the present study for several reasons, including (1) the use of technical grade resmethrin, rather than the more toxic formulation synergized with piperonyl butoxide; (2) a static exposure rather than constant concentrations via a flow through system and (3) the use of environmental conditions (such as temperature and salinity) favorable to lobsters.

Our data suggest that evaluation of phagocytosis using flow cytometry is a sensitive indicator of subtle sublethal effects of resmethrin, and that transient exposure to low, sublethal concentrations of resmethrin can affect lobster defense mechanisms. To our knowledge, this is the first report of immunomodulatory effects associated with exposure to resmethrin in any species. These effects were not immediate, appeared dose-dependant and exacerbated on repeated exposure. Immunotoxicity was observed at concentrations 10–100 times lower than those that failed to kill lobsters in the traditional 96-h LC50 assay. Whereas other authors claim that chronic toxicity to aquatic organisms will not be an issue for resmethrin (Rand 2002), the present demonstration of delayed, sublethal effects associated with exposure to resmethrin clearly warrants concern over the long-term effects of resmethrin on lobsters. In addition, the sublethal effects on chronic exposure of invertebrate and fish reproduction and larval growth did not occur below 0.59 μg/L (Rand 2002), whereas swimming performance was not altered at concentrations below 3.2 μg/L (synergized) and 6.3 μg/L (nonsynergized) (Paul & Simonin 1996), suggesting that either reproduction, larval growth and swimming performance are far less sensitive than the immune system to the toxic effects of resmethrin or that lobsters are far more sensitive than *Daphnia* and the fish species tested in other studies. Another interesting finding is the fact that phagocytosis was altered in lobsters exposed to 0.1 and 1 μg/L resmethrin 5 days after exposure (in the acute exposure study) but not 7 days after exposure (subacute exposure study). This suggests that either the effects are transient, or exacerbated by the stress associated with handling and water changes (on day 1, 3 and 5 in the acute exposure study, and on day 7, 14, 21 and 28 in the subacute study). The onset of effects on phagocytosis on exposure to 0.1 μg/L resmethrin on wk 3, the third time the lobsters are handled, as for day 5 in the acute study,

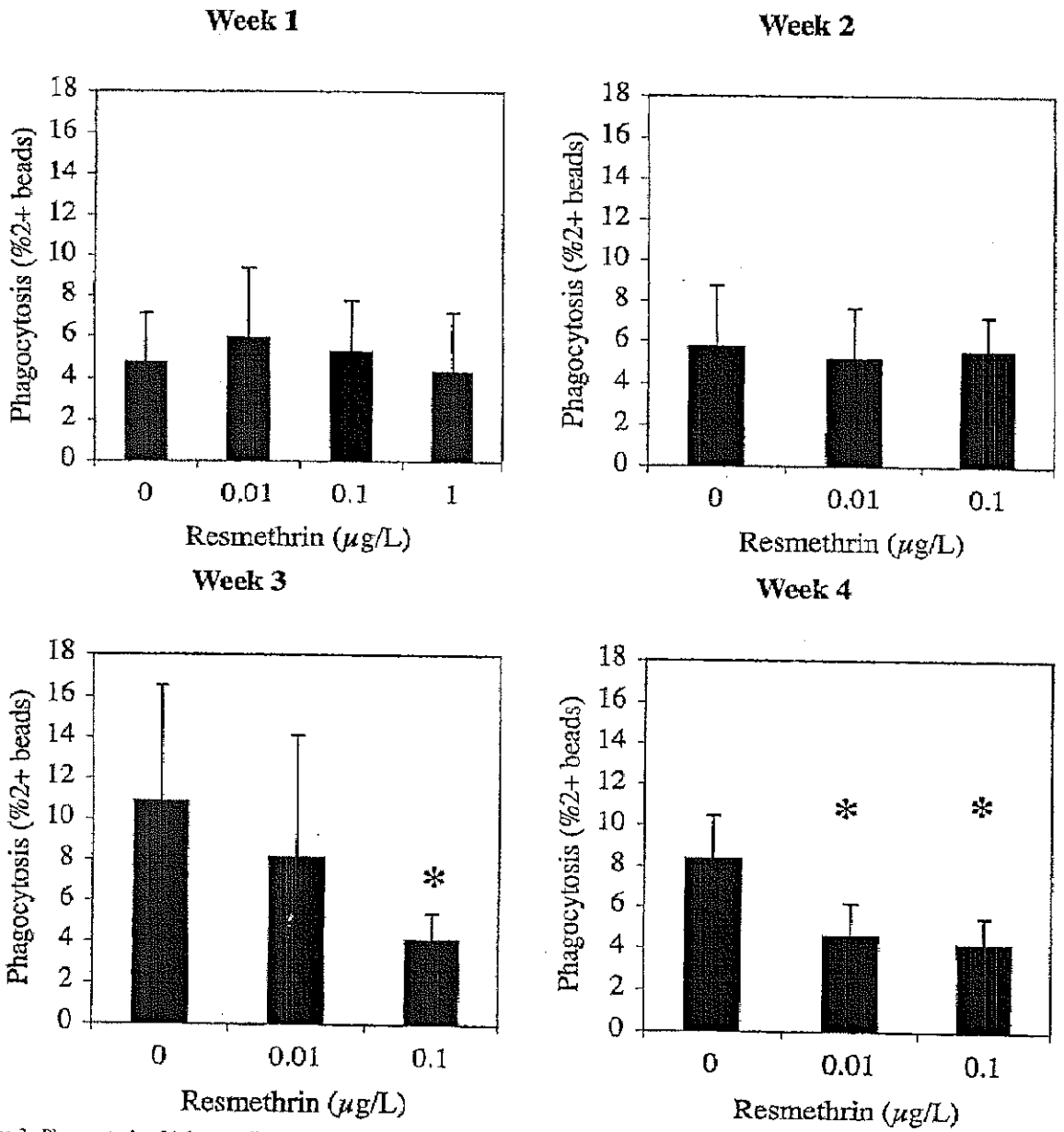

Figure 3. Phagocytosis of lobster cells as measured on day 7, 14, 21 and 28, in each case 7 days after a weekly exposure to three different concentrations of resmethrin. The highest concentration (1 μg/L) is not presented for wk 2, 3 and 4 because mortality in this group exceeded 50%. *significantly different from unexposed control ($P < 0.05$).

suggests that stress may in fact affect the immunotoxicity of resmethrin in lobsters. Similarly, the stress associated with repeated handling seems to result in a small but steady increase in phagocytosis in the control group for the acute subacute exposure, highlighting the importance of stressors on the immune system. The use of appropriate controls nevertheless allows the detection of the effects of resmethrin in time.

Mammalian studies comparing the relationship between immune functions and host resistance showed a good correlation between changes in the immune tests and altered host resistance, with no instances where host resistance was altered without affecting an immune test (Luster et al. 1993). We have documented immunomodulatory effects in lobsters on phagocytosis, reported as the most important defense mechanism in all phyla of the animal kingdom (van Oss 1986). Given that defective phagocyte function is an important cause of increased susceptibility to opportunistic pathogens in mammalian species (Rotrosen & Gallin 1987), and the above referenced data in mammalian immunotoxicology (Lus-

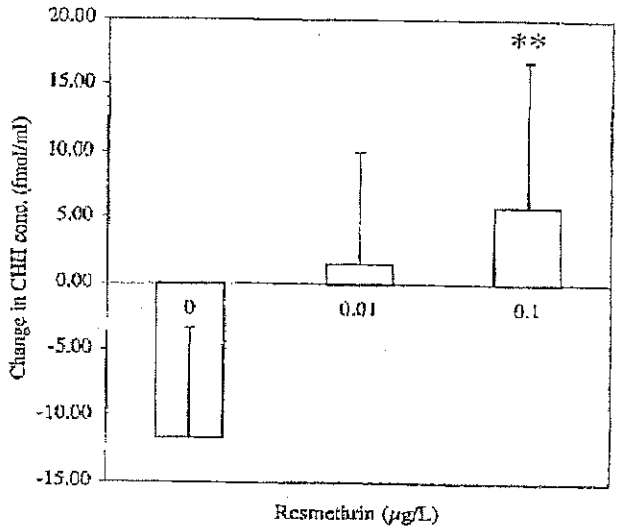

Figure 4. Changes (mean +/- SD) in hemolymph crustacean hyperglycemic hormone (CHH) after exposure for 4 wk at the indicated concentrations compared with values in the same animals prior to the start of the experiment. **significantly different from unexposed control ($P < 0.01$).

ter et al. 1993), it is likely that resmethrin-induced modulation of phagocytosis in lobsters could result in a significant decrease in lobster resistance to disease. It should nevertheless be noticed that the effects of resmethrin on other lobster defense mechanisms have not been evaluated, and the relative importance of and redundancy between different defense mechanisms in lobsters on encounter with pathogens are not well documented.

Elevated hemolymph glucose is required for lobsters to deal with any number of physiologic challenges. Because CHH regulates hemolymph glucose concentration, this hormone acts as a crustacean stress hormone. Elevations in hemolymph CHH have been shown to occur in lobsters following a number of environmental stresses. These include elevated temperature, hypoxia, osmotic stress (Chang et al. 1998, Chang et al. 1999) and parasitism (Stentiford et al. 2001).

An accepted definition of an endocrine disrupter is "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function" (Holmes et al. 1997). Our data demonstrate that resmethrin does indeed alter the circulating concentration of the hormone CHH. Whether this alteration results in adverse health effects remains to be seen. Resmethrin, a pyrethroid insecticide, has not previously been shown to be an endocrine disrupter in crustaceans. However, other pyrethroids have been implicated as potential endocrine disrupters through the use of vertebrate hormone binding assays (Kojima et al. 2004). The decline in the CHH concentrations in the control lobsters was likely caused by the acclimation of the animals to their aquaria after 4 wk.

The current study assessed the toxicity of resmethrin in lobsters in view of its use around Long Island Sound to control West Nile virus-infected mosquitoes and its potential role in the 1999 lobster die-off. We documented that adult lobsters did not appear more sensitive than other aquatic species to the acute lethal toxic effects of resmethrin but much more sensitive than other aquatic species to the sublethal effects of resmethrin, although those effects were documented on one immune function and hormone, not on reproduction, development and growth. However, to the authors' knowledge, no data have documented the actual exposure of lobsters to resmethrin on environmental application, and it is not known if the concentrations that we documented to be toxic to lobsters were ever found in their environment in the course of the die-off. It is therefore not possible to determine with any certitude the role of resmethrin in the 1999 lobster die-off, but the risk of sublethal effects should be further explored, because they appeared at low concentrations compared with malathion (De Guise et al. 2004a) and methoprene (De Guise et al. 2004b), the 2 other pesticides that were applied for the control of mosquitoes around Long Island Sound and under investigation for their possible role in the lobster die-off.

CONCLUSION

In conclusion, our results suggest that adult lobsters are not more sensitive than other aquatic species to the lethal effects of resmethrin, but are very sensitive to its sublethal effects compared with other aquatic species, at least the immune and endocrine endpoints tested. A modulation in immune functions could likely result in an increase susceptibility to infectious agents, and could have contributed to the mass mortality if exposure was sufficient.

ACKNOWLEDGMENTS

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Bioaccumulation and Metabolic Effects of the Endocrine Disruptor Methoprene in the Lobster, *Homarus americanus*[1]

ANNA N. WALKER,* PARSHALL BUSH,† JONATHAN PURITZ,‡ THOMAS WILSON,§ ERNEST S. CHANG,‖ TIM MILLER,** KENNETH HOLLOWAY,†† AND MICHAEL N. HORST††[2]

*Department of Pathology, School of Medicine, Mercer University, Macon, Georgia 31207
†Agricultural and Environmental Services Labs, University of Georgia, 2300 College Station Road, Athens, Georgia 30602
‡Department of Biological Sciences, Brown University, Providence, Rhode Island
§Department of Entomology, Ohio State University, Columbus, Ohio
‖Bodega Marine Laboratory, University of California, Davis, Bodega Bay, California 94923
**Darling Marine Center, University of Maine, Walpole, Maine 04573
††Division of Basic Medical Sciences, School of Medicine, Mercer University, Macon, Georgia 31207

SYNOPSIS. Methoprene is a pesticide that acts as a juvenile hormone agonist. Although developed initially against insects, it has since been shown to have toxic effects on larval and adult crustaceans. Methoprene was one of several pesticides applied to the Western Long Island Sound (WLIS) watershed area during the summer of 1999; the other pesticides were malathion, resmethrin, and sumethrin. These pesticides were applied as part of a county-by-county effort to control the mosquito vector of West Nile Virus. Subsequently, the seasonal lobster catches from the WLIS have decreased dramatically. The lethality of the pesticides to lobsters had been unknown. We studied the effects of methoprene while other investigators studied effects of the other pesticides. We questioned whether methoprene, through its effects on larvae, adults or both, could have contributed to this decline. We found that low levels of methoprene had adverse effects on lobster larvae. It was toxic to stage II larvae at 1 ppb. Stage IV larvae were more resistant, but did exhibit significant increases in molt frequency beginning at exposures of 5 ppb. Juvenile lobsters exhibited variations in tissue susceptibility to methoprene: hepatopancreas appeared to be the most vulnerable, reflected by environmental concentrations of methoprene inhibiting almost all protein synthesis in this organ.

Our results indicated that methoprene concentrates in the hepatopancreas, nervous tissue and epidermal cells of the adult lobster. Methoprene altered the synthesis and incorporation of chitoproteins (cuticle proteins) into adult postmolt lobster explant shells. SDS PAGE analyses of adult post–molt shell extracts revealed changes in the synthesis of chitoproteins in the methoprene-treated specimens, suggesting that methoprene affects the normal pathway of lobster cuticle synthesis and the quality of the post-molt shell. Although it is likely that a combination of factors led to the reduced lobster population in WLIS, methoprene may have contributed both by direct toxic effects and by disrupting homeostatic events under endocrine control.

INTRODUCTION

Juvenile hormone (JH) is a regulator of insect development. It modifies the response to the molting hormone, 20-hydroxyecdysone, at the molecular, cellular and organismal level. In larval insects, the presence of JH promotes larval-larvae molts, while its absence results in a pupal or adult molt (Riddiford, 1993, 1996). Methyl farnesoate (MF), synthesized in the mandibular organ of crustaceans, is the unepoxidated equivalent of Juvenile Hormone III (JH III) found in insects. The pesticide methoprene is a JH agonist (Staal, 1986; Wilson, 2004) and also mimics the action of MF in crustaceans (Laufer *et al.*, 1987).

During the warm weather months of 1999, concerns about the spread of the mosquito-borne West Nile Virus led to increased application of pesticide compounds in the New York City and Connecticut area. In mid-September of that year, the region experienced extremely heavy rainfall due to Hurricane Floyd. One month later, lobstermen in Western Long Island Sound (WLIS) began to report sightings of gravid female lobsters dying in the throes of abortive molts. The environmental concentrations of methoprene and of the other pesticides in WLIS during that time are unknown. The combination of circumstances and observations, however, led us to question whether methoprene, known to have been applied to the WLIS watershed, had disrupted the normal hormonal balance in ovigerous female lobsters and triggered molting at inappropriate times. The subsequent decrease since 1999 in the seasonal lobster catches would likewise indicate that one or more harmful events had increased morbidity and mortality among adult lobsters and their offspring. We undertook study of the acute effects of methoprene exposure on the survival of larval lobsters and to determine whether such an exposure would exert changes in the tissues of adult animals. The purpose of this paper is to provide a preliminary communication of our findings, as of January, 2004. A detailed report will be presented elsewhere.

MATERIALS AND METHODS

Culture

All larval experiments were conducted at the Darling Marine Center, University of Maine, Walpole,

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[2] E-mail: horstmn@mercer.edu

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ME. Gravid female lobsters were obtained from local fishermen and maintained in flowing seawater tanks equipped with an outlet screen to retain larvae. As they hatched, larvae were collected with a dip net and transferred to cylindrical tanks (0.3 × 1.5 m) equipped with a similar flow-through system. A large airstone at the bottom of each tank provided adequate suspension of the larvae throughout the entire water column. The ambient water temperature was 18°C; all experimental work with larvae was conducted at the same temperature. Larvae were fed live adult brine shrimp twice daily; the photoperiod was 12 L:12 D. Developmental stages of larvae were identified according to Factor (1995).

Acute exposure studies

All solutions for larval exposure studies were prepared using filtered seawater. S-Methoprene (Wellmark International, Schaumburg, IL) was dissolved and diluted in acetone (10 mM) prior to adding it to seawater; seawater used to maintain control animals contained an equivalent amount of neat acetone. Plastic development trays (Pfaff; B&H Photo, New York, NY) were half filled with various concentrations of methoprene in seawater (see Results) and aerated with a small airstone. Trays were maintained at 18°C under fluorescent light in a walk-in cold room. Plastic tackle boxes with 1 mm holes drilled in the bottom were used for exposure studies: each compartment (4 × 5 cm, with a depth of 3 cm seawater) housed one larval lobster. Thirty larvae were exposed to each concentration of pesticide tested (see Results). For postlarvae (Stage IV), 18 individuals were tested at each concentration. Larvae were fed adult brine shrimp *ad lib.* and were scored daily for molting and survival. Dead animals were removed from the trays immediately. Animals were maintained in the original volume of pesticide laden seawater for 7 days; thereafter animals were changed to normal seawater and the experiment was continued for an additional 7 days.

Bioaccumulation studies

Adult intermolt lobsters were equilibrated in the laboratory for at least 7 days. Individual lobsters (~570 g) were placed in plastic buckets containing 8 liter filtered seawater. Dilutions of methoprene (50 ppb final concentration) were made from a stock solution (5 mM) prepared in acetone. Control animals were treated with an equal volume of acetone in seawater. Exposures were conducted at 18°C for 4 hr. Thereafter, animals were anesthetized by packing in ice, sacrificed, and tissues were dissected and snap frozen in liquid nitrogen and stored at −80°C. Tissue samples were transported to the Pesticide Analysis Laboratory at the University of Georgia, Athens GA, where they were extracted and analyzed for methoprene content by gas chromatography-mass spectrometry (GC/MS). Sample preparation for GC-MS was essentially as described by Reed *et al.* (1977). Briefly, tissues (5 g) were homogenized in Na_2SO_4 (50 g) and ethyl acetate (300 ml) for 1 min in a motorized blender. After passage through glass fiber filter paper, the filtrate was concentrated using a rotary vacuum evaporator. The residue was re-dissolved in ethyl acetate: toluene (3:1), vortexed and clarified. The supernatant was defatted by gel permeation chromatography on BioBeads SX-3 (100–200 mesh; Bio-Rad); the eluting solvent was ethyl acetate: toluene (3:1). The sample was further purified by Fluorisil chromatography (serial elution with 6%, 15%, and 50% ether in hexane); material in the 15% fraction was turboevaporated and redissolved in 1 ml methylene chloride. Internal calibration standards were added and the extract was analyzed by gas chromatography-mass spectrometry as described by Noakes *et al.* (1999); the MS instrument was operated in selected ion monitoring (SIM) mode. Gas chromatographic conditions: column: RTX-5MS col (Restec, Inc.) 30 m by 0.25 mm ID megabore capillary column; oven temperature program: initial temperature = 70°C, initial hold 2 min; temperature programmed to increase at 20°C per minute to 210°C with a final hold of 10 min. Under these conditions, methoprene had a retention time of 29 min, phenanthrene d-10 retention time = 25.5 min; chrysene d-12 retention time = 33.9 min. Data were expressed as parts per million. The methoprene minimal detection limit varied dependent on sample size but was approximately 0.05 ppm (wet weight). A total of five animals have been exposed, dissected, extracted and analyzed thus far; here, we report representative data from a single animal.

In vivo *exposure studies*

Post-molt juvenile lobsters (3 cm carapace length) were exposed to 50 ppb methoprene in sterile seawater (1 liter) for 3 hr; controls were held in seawater only. Each animal was then injected through the dorsal carapace with 0.25 ml seawater containing 0.25 mCi Tran-^{35}S-Label (ICN Radiochemicals, Irvine, CA). The injection site was sealed with a drop of warm 1% agarose in seawater and the animals were held for 24 hr at 18°C. Animals were anesthetized and tissues dissected and placed on ice. As part of the preliminary studies presented here, a total of two animals have been exposed, dissected, extracted and analyzed thus far. Additional replicate experiments are underway.

In vitro *incorporation of ^{3}H D-glucosamine by explant cultures*

Postmolt juvenile lobsters were sacrificed 18 hr after ecdysis (molt stage A2) and the carapace was dissected on ice. After removal of muscle and connective tissue, the carapace was cut into 0.5 × 1 cm strips. About 4 strips were placed in each well of a 6-well plate and covered with 3 ml of explant culture media: Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 5% glucose, 0.49 M sodium chloride, 10% fetal bovine serum, and ABAM (penicillin/streptomycin/amphotericin B). After addition of methoprene (final concentration = 25 ppb) to experimental cultures or acetone carrier vehicle to controls, the samples were

pre-incubated at 18°C for 4 hours. After addition of ^{3}H D-glucosamine (50 μCi/ml; PerkinElmer Life Sciences; Specific activity = 30 Ci/mmol) to each well, the samples were incubated for an additional 16 hr. Afterward, epithelial tissue was removed from the cuticle with a plastic scraper. Samples of shell and epithelial tissue from control or methoprene treated wells were pooled, extracted and processed as described below. In this way, each individual postmolt animal served as its own control.

Tissue homogenization, differential centrifugation and extraction

Each tissue was homogenized with a Potter-Elvejhem homogenizer fitted with a Teflon plunger. Samples were homogenized ten strokes at 50% maximal rpm setting in Homogenization buffer: 20 mM Tris, pH 7.8, containing 0.4 M NaCl, 10 mM $MgCl_2$, 0.2 mM phenylmethanesulfonylchloride and protease inhibitor cocktail (Calbiochem). Shell samples were pre-extracted with 0.5 M EDTA, pH 7, containing PMSF and protease inhibitor cocktail for 12 hr at 18 °C. Homogenates were centrifuged (500 × g for 15 min) to remove cell debris and nuclei. The supernatant was centrifuged at 5,500 × g (15 min) to remove mitochondria and finally at 30,000 × g (45 min) to obtain a crude microsomal fraction ("16Kp") and cytosol (16Ks). The latter fraction was dialyzed against distilled water using 12 kDa cutoff dialysis membranes.

The 5,500 × g and 16Kp pellets were extracted with 8 M urea containing 0.2% dithiothreitol and PMSF. After centrifugation (10,000 × g for 15 min) the urea soluble supernatant was removed and dialyzed. After washing with 10 mM Tris, pH 7.4, the crude microsomes were collected by centrifugation (30,000 × g for 45 min). The microsomal pellet was then extracted with boiling 2% SDS in 10 mM Tris buffer, pH 7.4 for 5 min. The samples were filtered through Nytex screen and the residue was washed sequentially with water, ethanol and acetone. The final shell residue was air dried at room temperature to constant weight. Radioactivity in all fractions was measured and expressed as cpm/mg sample.

SDS-PAGE procedures

Samples of control and pesticide treated fractions were prepared for SDS-PAGE by boiling in 10 mM Tris, pH 7.0 containing 2% SDS, 15% glycerol, 0.001% brom phenolblue and 0.2% dithiothreitol for 3 min. Samples (15 μl) were applied to either precast 4–20% gradient gels (PageR Gold, Cambrex) or 10% acrylamide gels and separated according to Laemmli (1970). At the completion of the experiment, gels were either fixed and stained for total protein with Sypro Ruby or Coomassie Blue or electroblotted to PVDF membranes using a semi-dry technique. The membranes were then blocked with 5% Blotto (Bio-Rad) in Tris buffered saline (TBS) at 4°C overnight. Blots were probed with biotinylated Tomato Lectin (Pierce Chemical Co) followed by detection with Streptavidin-HRP conjugate (Dako). After final washing, the blots were treated with ECL Plus reagent (Amersham) and positive bands detected using Probe Plus X-ray film (Pierce). After exposure for 1–10 min, films were developed using an automated (Xomat) processor.

RESULTS

Stage II larvae

Initial observations of Stage II larvae exposed to environmental concentrations of methoprene had indicated that exposed animals experienced a consistently higher mortality rate than did the controls. Accordingly, Stage II larvae (30 at each concentration) were maintained in seawater trays containing 0.1, 0.5, 1 and 10 ppb methoprene respectively for 72 hr. During that time period, the mortality rates were essentially the same as that of the controls for animals in the 0.1 and 0.5 ppb trays (approx. 14%). In contrast, at 1 ppb methoprene, the mortality rate was 30% and at 10 ppb, the mortality rate was 86% after 72 hr of exposure.

Stage IV larvae

Overall these animals appeared to be more resistant to the effects of methoprene than were the younger larval forms. They did not experience increased rates of mortality when exposed to methoprene. Instead, methoprene accelerated the frequency of molting in these animals.

Eighteen animals were studied at each concentration. During three days of methoprene exposure at 1 ppb concentration, no animals molted; no control animals molted during this time either. At concentrations of 5 ppb and above, however, the number of molts increased with an average of one third of the animals undergoing molts.

At day three, there were no deaths among the animals exposed to methoprene concentrations below 50 ppb. At 50 ppb, two animals suffered molt-related deaths (11% mortality).

Juvenile lobsters

These animals were exposed to 50 ppb methoprene for 3 hr and then injected with Tran^{35}S-Label. Twenty-four hours later, the hepatopancreas, gills and muscle tissue were removed from each animal and mitochondrial, crude microsomal and cytosolic fractions of each tissue were prepared. As assessed by the incorporation of Tran^{35}S-label into microsomal and cytosolic fractions, the hepatopancreatic tissue from exposed animals suffered greater than 90% inhibition of protein synthesis. The gill tissue exhibited a 50% decrease in protein synthesis while muscle tissue was largely unaffected.

Adult studies

As part of this preliminary study, we exposed a total of three adult animals to 50 ppb methoprene for 3 hr. The pesticide was concentrated in the following tis-

TABLE 1. *Bioaccumulation of methoprene in specific tissues after acute exposure of adult lobsters.*

Sample description	Sample weight (g)	Methoprene concentration (ppm)
Calibration standard	30	3.97
Hepatopancreas	10.39	1.55
Gills	7.52	0.14
Epithelial cells	0.81	6.17
Muscle, abdominal	6.3	0.16
Gonad	1.08	5.18
Stomach	7.17	n.d. @ <0.14
Connective tissue	1.40	n.d. @ <0.71
Eyes	0.35	28.83
Heart	0.70	n.d. @ <1.43

As described in Methods, adult lobsters (molt stage C4) were exposed to 50 ppb methoprene at 18°C for 4 h. Animals were removed from exposure medium, briefly drained then anesthetized on ice for 30 min. Thereafter, tissues were dissected and frozen in liquid nitrogen. Samples were weighed, extracted, and analyzed for methoprene content by GC-MS (see Methods). Results are expressed as concentration of methoprene in parts per million in each of the tissues sampled; n.d., non detectable at or less than the value given. See text for details.

sues: hepatopancreas, gonad, epidermis, and neural tissue (ganglia and eye stalk; See Table 1).

Explant studies

The advantage of using the explant method is that control tissue and experimental tissue are derived from the same animal, thereby obviating inter-animal variation in molt stage or metabolic status.

Incorporation of ^{3}H-glucosamine. Total radioactivity incorporated into 18 hr (molt stage A_2) post-molt epithelial cells in the presence of methoprene ("experimental") was 38% of that incorporated into control epithelium. Incorporation into the cytosolic fraction from the experimental was approximately equal to that of the control. In the 5,500 × g pellet (containing membrane fragments and mitochondria), however, there were marked differences between the 25 ppb treated specimens and the controls. These pellets were first extracted with urea; the experimental contained 52% less radioactivity than control. The urea insoluble residues were then extracted with SDS; the experimental fraction contained 92% less radioactivity than the control.

Incorporation of Tran^{35}S-Label. The shells from methoprene exposed and control explants were sequentially extracted and total non-dialyzable radioactivity in each fraction was determined. The fractions extracted with EDTA were approximately equal. The buffer extracted fraction of the experimental, however, contained 50% less radioactivity than the control, while the urea extracted fractions were approximately equal. The SDS extracted experimental fraction contained 30% less radioactivity than the control.

SDS PAGE analysis

Analysis of solubilized shell fractions from explant cultures by SDS-PAGE indicated subtle alterations in the total protein profile as revealed by Sypro Ruby staining. As shown in Figure 1a, methoprene treatment caused decreased levels of several protein components with molecular weights of 40, 60 and 75 kDa. On the other hand, when Western blots of duplicate gels were probed with Tomato Lectin (TL, which binds chitin oligosaccharides), differences were observed (Fig. 1b), most notably in the buffer soluble fractions. After methoprene treatment, TL positive components were either absent or reduced at 6, 25, 40, 80, 160 and 240 kDa, suggesting a major effect on an early stage in the synthesis pathway (Horst *et al.*, 1993). Increased levels of specific bands (40, 80 and 160 kDa) were observed in the urea soluble fraction. The SDS soluble fraction appears to have minor quantitative changes as a result of methoprene treatment.

Analysis of the cellular layer of the integument (which is predominantly epithelial cells) by 10% SDS PAGE showed marked differences in the total protein profiles of all extracted fractions (Fig. 2a; changes indicated by stars). Major protein alterations were observed at 45, 100 and 150 kDa. When Western blots of duplicate gels were probed with TL, we again observed alterations in TL positive proteins in the specimens (Fig. 2b). In the cytosolic fraction (lanes 1,2), methoprene treatment caused a shift of TL positive bands from high molecular weight (150–250 kDa) to low molecular weight components (20–40 kDa). SDS extracts of the 16,000 rpm pellet (lanes 5,6) showed decreased levels of 70 kDa material and increased 45 kDa material as a result of methoprene treatment. Analysis of the fractions of the 5,500 × g pellet extracted with urea and then with SDS showed remarkable alterations, but of opposite patterns. The urea soluble fraction showed marked increases in TL positive bands at 120 and 130 kDa as well as clusters of less intense bands at 90, 60, 45 and 36 kDa. By contrast, the SDS soluble material contained fewer bands following methoprene treatment in the higher molecular weight range (60–100 kDa).

DISCUSSION

The effects of JH and JH analogs in insects have been studied extensively (Wyatt and Davey, 1996; Riddiford, 1996; Hammock and Quistad, 1981; Berger *et al.*, 1992; Jones, 1995). In a 1996 review, Riddiford noted that JH allows molting in response to ecdysteroids but alters the switch of gene expression necessary for insect metamorphosis. Methoprene, acting as a JH agonist, has been shown to disrupt the metamorphic reorganization of the insect central nervous system, the salivary glands and the musculature (Restifo and Wilson, 1998). Other investigations indicated toxicity of methoprene to crustacean larvae. Templeton and Laufer (1983) found that methropene at concentrations known to interrupt insect development also interfered with the larval development of *Daphnia magna.* Other investigators found that environmental concentrations of methoprene affected molting, fecundity and the production of male off-

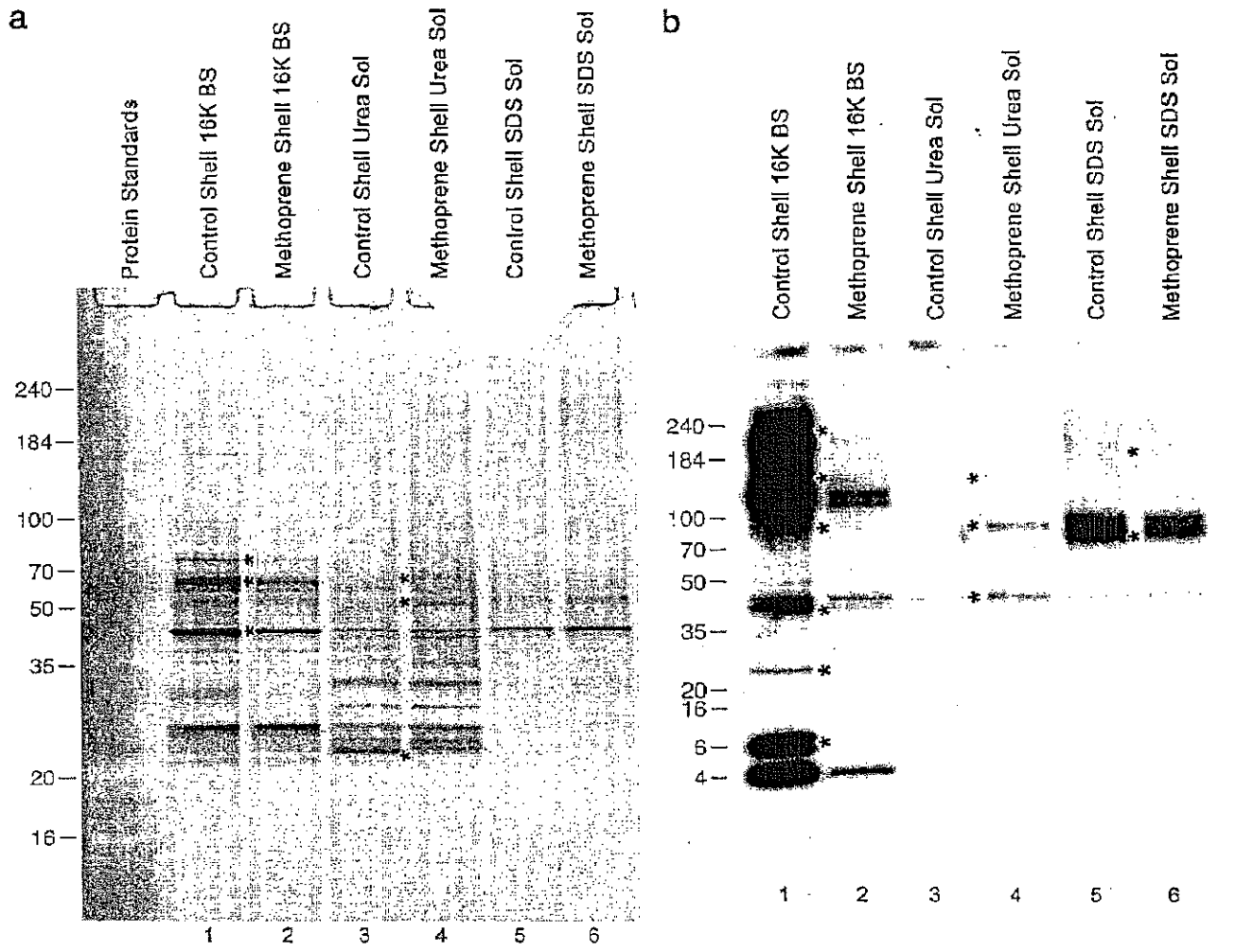

FIG. 1. a. Gradient SDS-PAGE analysis of solubilized fractions from post-molt lobster cuticle. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4 and 6); control explants are shown in lanes 1, 3 and 5, as described in Materials and Methods. Shell proteins were extracted with buffer (lanes 1 & 2), 8 M urea (lanes 3 & 4) and finally boiled in 2% SDS (lanes 5 & 6). After electrophoresis, the gel was fixed and stained with Sypro Ruby. Position of prestained molecular weight markers are indicated at left of lane 1; molecular mass is reported as kDa. Changes in protein components caused by methoprene exposure are indicated by dots in the buffer soluble and urea soluble fractions. b. Western blot analysis of solubilized fractions from post molt lobster cuticle. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4 and 6); control explants are shown in lanes 1, 3, and 5. All explants were extracted and separated by SDS-PAGE; as described in Materials and Methods. Proteins were transferred to PVDF membrane by semi-dry blotting and probed with biotinylated Tomato lectin (TL). Binding was detected with streptavidin-HRP followed by enhanced chemiluminescent detection (ECL); the exposure time was 1 min. Position of prestained molecular weight markers blotted to the PVDF membrane are indicated at left of lane 1; molecular mass is reported as kDa. Changes in TL-positive chitoprotein bands caused by methoprene exposure are indicated by dots in the buffer, urea and SDS soluble fractions.

spring in this species (Olmstead and LeBlanc, 2001*a*, *b*, 2003; Peterson *et al.*, 2001). Similar results have been observed in all life stages of salt marsh copepods (Bircher and Ruber, 1988).

Regarding decapod crustaceans, McKenney and Matthews (1990) showed that concentrations of 1 ppm methoprene were uniformly fatal to the larvae of *Palaemonetes pugio* while 0.1 ppm greatly reduced the number of larvae able to complete metamorphosis. Christiansen *et al.* (1977) reported that 1 ppm methoprene was acutely toxic to the larvae of *Rhithropanopeus harrisii*, but these investigators did not find harmful effects at 0.1 ppm methoprene under normal conditions of temperature and salinity. Other studies, however, revealed that this lower concentration did produce adverse effects if coupled with sub-optimal temperature and salinity (Payen and Costlow, 1977; McKenney and Matthews, 1990; Celestial and McKenney, 1994). Additional work by McKenney and Celestial (1993) on larval *Palaemonetes pugio* showed

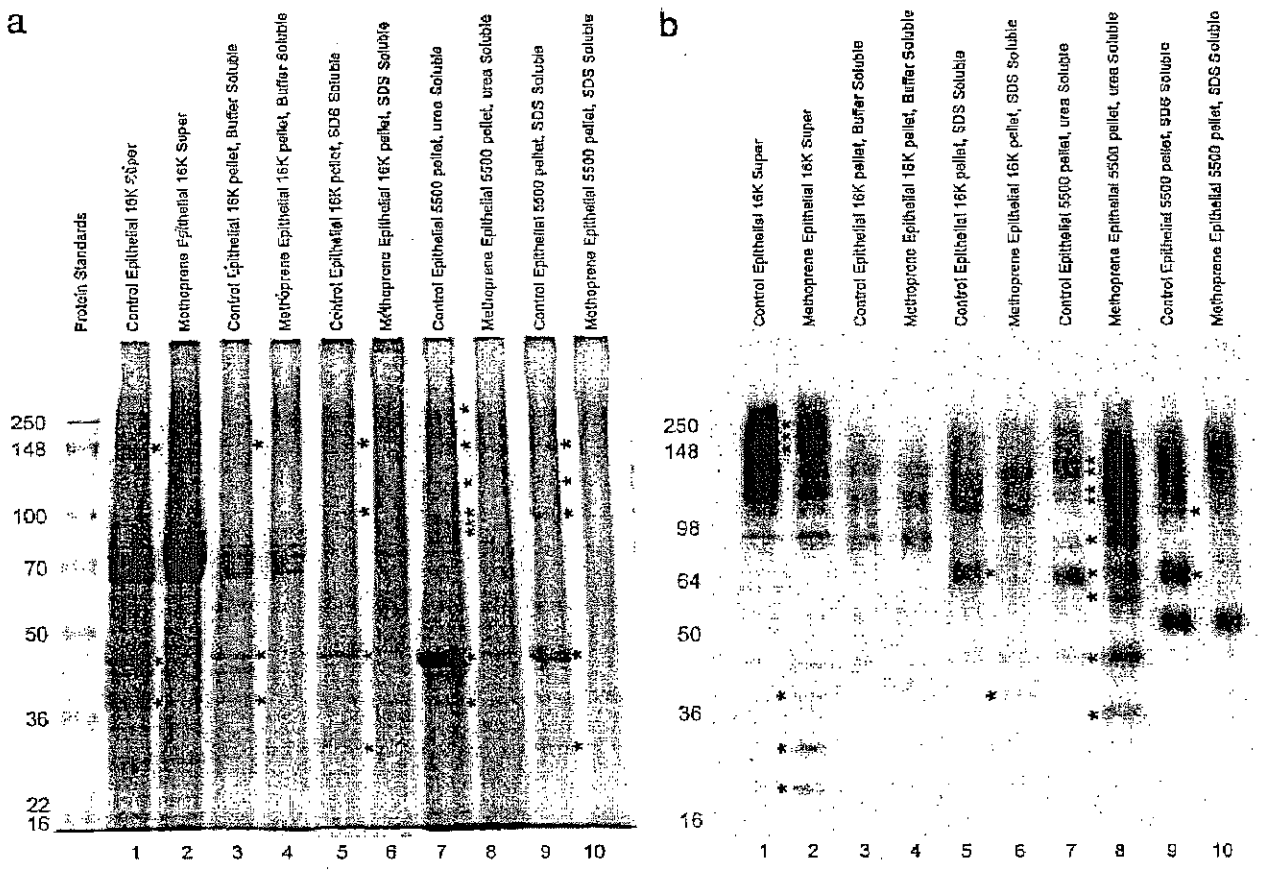

FIG. 2. a. 10% SDS-PAGE analysis of solubilized fractions from postmolt lobster cuticular epithelial cells. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4, 6, 8, & 10); control explants are shown in lanes 1, 3, 5, 7 & 9. After homogenization of cells, low speed (5,500p) and high speed pellets (16Kp) were obtained and proteins were extracted with buffer, 8 M urea and boiling 2% SDS as described in Methods. Samples were subjected to electrophoresis on 10% gels, fixed and stained with colloidal Coomassie Blue. Samples are identified by lane in the figure. For details, see Methods. The position of prestained molecular weight markers are indicated at left of lane 1; molecular mass is reported as kDa. Changes in the protein profiles of each sample caused by methoprene exposure are indicated by stars. b. Western blot analysis of solubilized fractions from post-molt lobster cuticular epithelial cells. Samples were prepared and separated on 10% gels as described for Figure 3. After electrophoresis, proteins were transferred to PVDF membrane by semi-dry blotting and probed with biotinylated Tomato Lectin (TL). Binding was detected with streptavidin-HRP followed by enhanced chemiluminescent detection (ECL); the exposure time was 5 sec. Lane identities are described in Figure 2a legend. Position of prestained molecular weight markers blotted to the PVDF membrane are indicated at left of lane 1; molecular mass is reported as kDa. Changes in the protein profiles of each sample caused by methoprene exposure are indicated by stars.

that 8 ppb methoprene affected growth and inhibited the completion of metamorphosis.

McKenney and Celestial (1996) found that the juvenile forms of the estuarine mysid *Mysidopsis bahia* suffered complete mortality when exposed to methoprene concentrations of 125 ppb and exhibited diminished size and subsequent fecundity when reared in concentrations of methoprene as low as 8 ppb. Chu *et al.* (1997) found an LD_{50} of 0.34 ppm methoprene at 48 hr for the freshwater cladoceran *Moina macrocopa*; they also found that levels of 5–10 ppb actually stimulated reproduction. Ting *et al.* (2000) found that exposure of the naupliar forms of the harpacticoid copepod *Tigriopus californicus* to 10 ppb methoprene resulted in subsequent disruption of mate recognition, a process known to be under endocrine control. Studies by Ahl and Brown (1990) demonstrated delayed ecdysis and molt-related mortality in brine shrimp larvae (*Artemia* sp.) exposed to 0.3 ppm methoprene.

These last investigators also found that methoprene had a stimulatory effect on Na/K ATPase activity in *Artemia* (Ahl and Brown, 1991). They suggested that the mechanism of action involved direct binding of the pesticide to regulatory sites on the enzyme causing changes in conformation. Due to its hydrophobic structure, methoprene could also lodge in membranes and modify the lipid environment adjacent to enzymes leading to altered conformation and therefore activity. Subsequent studies by Lovett *et al.* (2001) in the green

crab *Carcinus maenas* indicated that MF itself plays a role in osmoregulation.

In our studies of the blue crab (*Callinectes sapidus*), we found that methoprene, in keeping with its hydrophobic nature, could penetrate the investment coat of the blue crab embryo and localize in lipovitellin. Exposure of the embryos to environmental concentrations of methoprene resulted in an overall reduction in the number of successful hatchings and in lethargic swimming behavior on the part of the newly hatched survivors (zoea larvae). Moreover, in later larval forms (megalopae), methoprene delayed the molt to the first crab form and resulted in death of 80% of larvae after exposure for 10 days. It was our conclusion that blue crab larvae exposed to methoprene could either die as a direct result of metamorphic disruption or be compromised in their ability to swim such that they were vulnerable to increased predation (Horst and Walker, 1999).

Although we did not observe altered swimming behavior, we did find that low levels of methoprene had adverse effects on lobster larvae. Methoprene concentrations of 1 ppb or higher significantly affected survival for Stage II larvae. Stage IV larvae were more resistant, but did exhibit significant increases in molt frequency beginning at exposures of 5 ppb methoprene.

Our studies of juvenile lobsters revealed variations in tissue susceptibility to the effects of methoprene. The hepatopancreas appears to be the most vulnerable, with environmental concentrations of methoprene inhibiting almost all protein synthesis in this organ. The hepatopancreas is critical to homeostasis in crustaceans, being involved in digestion of food, absorption of nutrients, production of hemolymph proteins and host defense against infectious agents. The *in vivo* consequences of hepatopancreatic compromise could include, therefore, both increased morbidity and mortality of juvenile lobsters.

Although phylogenetically related, insects and crustaceans have many obvious anatomic and physiologic differences. One difference is the necessity of many crustaceans to molt not only during embryonic and larval stages, but also to continue to molt throughout adulthood. Studies by Laufer *et al.* (1987), Chang (1993) and others have indicated that MF influences the crustacean molt in part by increasing the synthesis of the molt-related hormone, ecdysone. Laufer *et al.* (1998) have also shown that MF stimulates ovarian maturation in the crayfish *Procambarus clarkii*.

The increased frequency of molts in the stage IV larvae and the historical observation of berried females dying while attempting to molt raise the possibility that methoprene could be responsible for endocrine disruption in larval and adult lobsters. Other investigators have shown an interplay between MF and ecdysone in other crustaceans. In 1975, Demeusy suggested that large premolt increases in the hemolymph ecdysteroid titer were due to an ecdysiotrophic action of methyl farnesoate. Then, in 1977, Hinsch demonstrated that, during the premolt, the mandibular organ in *Libinia emarginata* undergoes ultrastructural changes indicative of increased synthetic activity. In *Cancer magister*, Tamone and Chang (1993) showed that ecdysteroid synthesis by Y organs is increased both by incubation with mandibular organ-conditioned medium or with methyl farnesoate. If methoprene is acting as an MF analog, it is reasonable to suggest that methoprene could affect the synthesis of ecdysone, and accordingly influence the timing and frequency of molts. Molting is a stressful and vulnerable time for all crustaceans, larval and adult; thus, increased numbers of molts lead to increased periods of vulnerability to predation, trauma and infection. Moreover, our studies also indicate that methoprene has an effect on the quality of the post-molt shell.

Our studies in blue crabs revealed that methoprene interrupted chitin production in the adult postmolt blue crab as evidenced by decreased incorporation of ^{3}H-glucosamine into methoprene-treated explant cuticle. We also found an effect on protein synthesis as shown by diminished incorporation of radiolabeled amino acids into the explant shells (Horst and Walker, 1999). In the present investigation, we observed that methoprene alters the synthesis and incorporation of both buffer and urea soluble chitoproteins into adult postmolt lobster explants. Presumably, these two fractions represent intermediate stages of glycosylation.

In the present study, SDS PAGE analyses of adult post–molt shells revealed changes in chitoprotein expression in the methoprene-treated specimens. These findings suggest that methoprene blocks the normal pathway of crustacean cuticle synthesis (Horst *et al.*, 1993). Crustacean cuticular chitin is a glycoconjugate complex containing chitin oligosaccharide chains covalently attached to protein. Following biosynthesis, precursors may be exocytosed and incorporated into the postmolt cuticle by crosslinking reactions. In our studies on blue crabs, we established that the SDS insoluble residue of the cuticle contained macromolecular chitin (Horst and Walker, 1999). Such material represents the final stage in the crustacean chitin synthesis pathway; its production by blue crab explants was affected by methoprene when experiments were conducted for a prolonged period of time, *e.g.*, 24–48 hr. The present study on lobster explants included only short-term exposures to methoprene wherein the precursor pool proved to be more dramatically affected than was the formation of final product. Long-term exposure studies are planned.

Bioaccumulation of pesticides has been reported in other crustaceans (Kusk, 1996; Bhavan *et al.*, 1997; Bhavan and Geraldine, 2002; Robinson *et al.*, 2002). The pesticides appear to enter with food into the digestive tract, and are absorbed in the hepatopancreas. There is little information on subsequent transfer of pesticides to other tissues in crustaceans. Our results indicate that methoprene concentrates up to 125-fold over the surrounding sea water in the hepatopancreas, gonadal tissue, nervous tissue and epidermal cells of

the adult lobster. Crustacean epidermal cells are the site of chitoprotein synthesis for shell production. The apparent preferential localization of methoprene into epithelial tissue helps explain the alteration in the chitoprotein expression observed in the exposed explants. Additional bioaccumulation data are presented elsewhere (Walker and Horst, 2004).

Because of the time of the year, Stage II and IV lobster larvae as such would not have been present to experience detrimental environmental influences in WLIS immediately after Hurricane Floyd. Their parents, however, would have been. It has been shown that the quality of the maternal environment influences the survival and success of offspring (Waddy *et al.*, 1995). Methoprene, being lipid soluble, would be expected to accumulate on/in organic debris on the sound bottom. Adult lobsters frequently bury themselves in mud, thereby facilitating their exposure to lipophilic pesticides. Moreover, adult lobsters could also suffer chronic systemic methoprene exposure due to slow release of pesticide deposited in lipid-rich tissues. Even if a berried female had not produced eggs at the time of the contamination, methoprene could concentrate in her lipid-rich tissue and then contaminate her eggs through localization in the lipid-rich lipovitellin. Subsequent embryonic and larval forms could suffer direct toxicity as well as endocrine disruption due to methoprene. Although it is likely that a combination of factors led to the reduced lobster population in WLIS, methoprene may well have played a significant part in this economic and environmental calamity.

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